

## Polyphenolic Profiling of Croatian Propolis and Wine

Marica Medić-Šarić<sup>1\*</sup>, Mirza Bojić<sup>1</sup>, Vesna Rastija<sup>2</sup> and Josipa Cvek<sup>3</sup>

<sup>1</sup>Department of Medicinal Chemistry, Faculty of Pharmacy and Biochemistry, University of Zagreb, A. Kovačića 1, HR-10000 Zagreb, Croatia

<sup>2</sup>Department of Chemistry, Faculty of Agriculture, J. J. Strossmayer University of Osijek, Kralja Petra Svačića 1d, HR-31000 Osijek, Croatia

<sup>3</sup>Agency for Medicinal Products and Devices of Croatia, Ksaverska cesta 4, HR-10000 Zagreb, Croatia

Received: August 16, 2012  
Accepted: November 21, 2012

### Summary

Polyphenols are ubiquitous natural compounds that show chemopreventive, cytostatic, immunomodulatory, bacteriostatic/bactericidal, antifungal, anti-inflammatory, antioxidant and many other pharmacological activities. Propolis, wine and many medicinal plants used in everyday life as functional food present rich sources of polyphenols. In this paper we focus on their production, chemical analysis (spectrophotometry, HPLC, HPTLC, GC/MS, *etc.*) of flavonoids and phenolic acids, all of which enable authentication and geographical traceability of propolis and wine. This represents the basis for quality control and regulatory framework for any dietary supplement claiming to have beneficial health effectiveness.

*Key words:* flavonoids, propolis, wine, geographical traceability

### Introduction

The market of dietary supplements is primarily influenced by the demands of the consumer. One cannot be immune to the fact that half of the western world population dies from cardiovascular diseases and one quarter from cancer. What is postulated as major cause of cancer is oxidative stress and free radicals. Thus, antioxidants are nutraceuticals of choice that the consumer will use as over-the-counter product, often on a person's own initiative. Producers of nutraceuticals react quickly to these demands providing huge variety of products claiming to have antioxidant power. Major antioxidant effect is attributed to natural compounds such as flavonoids and phenolic acids. Driven by these facts, in this minireview, we have gathered the information on polyphenols in propolis and wine.

The overview of propolis as a rich source of polyphenols, with the focus on tincture as the most common

formulation is given. Special attention is devoted to quality control, providing guidelines for a certificate of analysis. Due to their specific composition, analysis of polyphenols in wines enables profiling based on grape varieties (fingerprinting).

As polyphenols are present in numerous plants that are emerging on the market, special attention is addressed to regulation of these products, mainly focusing on current directives of the European Union and International Conference on Harmonisation (ICH) guidelines.

### Characterisation and Standardization of Propolis

#### *Physical characteristics of propolis*

Propolis is the resinous substance collected by honeybees (*Apis mellifera* L.) from various plant sources. The colour of propolis ranges from yellow to dark brown depending on the origin of the resins. It has characteristic

\*Corresponding author; Phone: ++385 1 4818 304; Fax: ++385 1 6394 477; E-mail: [mmedic@pharma.hr](mailto:mmedic@pharma.hr)

aromatic smell and bitter to almost sweet taste. Propolis is sticky at and above room temperature, while at lower temperatures it becomes hard and very brittle. It can be homogenized by grinding to a fine powder after storage in a refrigerator or freezer for a few hours (1–3). The most common solvents used for commercial extraction of propolis are ethanol, glycerol and water. Other solvents like propylene glycol (for improved dissolution in water-based emulsions, production of nasal or oral sprays), acetone (for production of shampoos and lotions) and vegetable oils (olive, almond and linseed oil) can also be used in propolis processing. For chemical analysis a large variety of solvents (methanol, chloroform, hexane, *etc.*) may be used in order to extract various fractions. Although a stable product, propolis should be stored in airtight containers at temperature lower than 15 °C and protected from light (4).

Propolis-based products are marketed as various food supplements and medicinal cosmetic preparations (alcoholic and non-alcoholic solutions, oil drops, oral and nasal sprays, ointments, creams, tablets and capsules), often enriched with herbal extracts. Commercial uses of propolis are based on preparations made from primary liquid extracts, mostly propolis tinctures. After reduction or elimination of the solvent by lyophilisation (freeze drying) or vacuum distillation, propolis tincture could be modified to different dosage forms. Because of its antioxidant and antimicrobial activities, propolis has found applications in food production as a preservative (4).

#### *Standardization and quality control of propolis*

Among the substances with biological activity, none contributes more to the observed effects of propolis than plant phenolics (mostly phenolic acids and flavonoids). Phenolic composition is greatly variable depending on the local flora and season of collection. Even propolis samples taken from within a single colony can vary, making controlled clinical tests difficult, and the results of any given study cannot be reliably extrapolated to propolis samples from other areas. Thus, the chemical characterization of the sample is a prerequisite for the future use of propolis in modern medicine. Determination of clear criteria for quality control and standardization of extraction procedure are also essential (5).

According to the literature, different extraction techniques are applied for the extraction of the biologically active constituents of propolis. The common method employed is traditional maceration extraction with alcohol, leaving the alcohol-insoluble or wax fraction (6). Using 70 % (by volume) ethanol as generally accepted extraction solvent, Trusheva *et al.* (3) compared the efficiency of three extraction methods: traditional maceration, microwave-assisted extraction (MAE) and ultrasound extraction (UE). In all the tested techniques, the fraction of solvent used (1:10 or 1:20 *m/V*) was not significant for the extraction yield. This finding demonstrates that the use of solvent/propolis fraction larger than 1:10 *m/V* is unnecessary, leading only to solvent and energy losses. Compared to the time-consuming traditional maceration (10 days), MAE (2 cycles of irradiation for 10 s) and UE methods (30 min of sonication) provided high extraction yield with short time frames and less labour. However,

MAE method was found to be of low selectivity due to the extraction of high amounts of unwanted wax. In addition, longer irradiation times (3 cycles) resulted in a decrease of the percentage of extracted active components, presumably owing to degradation processes like oxidation. Thus, the authors concluded that the UE of bioactive phenolics and flavonoids from propolis is the most efficient extraction method (3). In another experiment, testing the influence of maceration time with 70 % (by volume) ethanol as solvent (1:5 *m/V*), it was shown that prolonged extraction periods did not necessarily result in richer propolis extracts. In fact, their qualitative composition remained practically the same over a very broad period of maceration time (20 days up to 1 year) (7). Observing the maceration of propolis with 96 % (by volume) ethanol (1:5 *m/V*) for 30 days and with solvent renewal every 7 days revealed that the solvent saturation was not a limiting factor for extraction efficiency (8). It was also observed that extraction with 70 % (by volume) ethanol resulted in wax-free tinctures with approx. 20 % more total phenolic substances compared to absolute ethanol (9). According to European Pharmacopoeia, beeswax (*Cera alba* and *Cera flava*) is partially soluble in hot 90 % (by volume) ethanol (10). The term 'partly soluble' is used to describe a mixture where only some of the components dissolve (the common components of waxes are alkanes, esters, ketones, and free carboxylic acids and alcohols) (10). In the above presented experiments propolis samples were ground and homogenized prior to extraction in order to increase the surface of contact with the solvent.

Besides the quantification of phenolics, biological study carried out with propolis extracts is another concept for extraction method evaluation. Park and Ikegaki (11) evaluated antimicrobial, antioxidant and anti-inflammatory activities of propolis extracts (2:25 *m/V*) that were prepared using water and various volume fractions of ethanol (10 to 95 %) as solvents and by shaking at 70 °C for 30 min. The 60 to 70 % ethanol extracts of propolis inhibited microbial growth (tested against *Staphylococcus aureus*) efficiently and then the inhibition decreased with increased ethanol volume fractions. The 70 and 80 % (by volume) ethanolic extracts had the greatest antioxidant activity and 80 % (by volume) ethanol extract strongly inhibited hyaluronidase activity. Samples of water extract and 10 and 20 % (by volume) ethanol extracts showed the lowest antioxidant activity and did not inhibit microbial growth. Chemical analysis proved that the highest concentration of flavonoids was liberated from the propolis when using 80 % (by volume) ethanol (11).

The first attempt to standardize propolis was done by Vanhaelen and Vanhaelen-Fastré in 1979 (12). They proposed six parameters for quality control of propolis: calcination residue, residue insoluble in water and in organic solvents, microscopic analysis of the insoluble residue, saponification number, chromatographic identification of five phenolic acids and three flavonoid aglycones, and antibacterial test (13). This proposal combines biological test and the identification of some phenolics, but no quantification is performed although this might be of great importance with respect to the variability of propolis composition. Despite the chemical differences, it is well

known that samples of different geographical origin and chemical composition usually demonstrate similar biological activity, particularly antimicrobial. For instance, the content of total flavonoids in European samples is usually 20 to 30 %, whereas in Brazilian samples it is about 5 %. However, some non-phenolic compounds, like diterpenic acids, have been identified as active constituents of Brazilian propolis. Furthermore, propolis samples of tropical origin do not contain caffeic acid phenethyl ester (CAPE), which is one of the most important active components. Because of such diversity in its composition, universal chemical characterisation of propolis is not possible, but only determination of its chemical type according to the plant source. Characteristic active substances or marker compounds defined in this way should be quantified and used for quality control of propolis-based products (13). Chromatographic techniques are most frequently used for determination of propolis chemical profile. In thin layer chromatography (TLC), a classical stationary phase of silica gel is widely used to separate more non-polar flavonoids (such as flavonols and isoflavonoids) with different mobile phases as eluent: ethanol/water (55:45 by volume), petroleum ether/ethyl acetate (70:30 by volume), petroleum ether/acetone/formic acid (70:20:10 by volume), chloroform/ethyl acetate (60:40 by volume), toluene/chloroform/acetone (40:25:35 by volume), *n*-hexane/ethyl acetate/acetic acid (58:39:3 by volume) and chloroform/methanol/formic acid (89:6:5 by volume). Visualization is performed at short (254 nm) and long wavelength UV light (366 nm), and in some cases by spraying with different reagents (1). Gas chromatography coupled to mass spectrometer (GC/MS) allows detailed molecular mass and structural information together with the identification of compounds. Propolis, however, contains components that are not volatile enough for direct GC/MS analysis even upon derivatization. As confirmed by a number of published papers, the most popular and reliable analytical technique for propolis characterisation is the high-performance liquid chromatography (HPLC) coupled with UV/VIS and/or fluorescence detector and MS (14,15).

As proposed by Bankova (16), chemical profile of propolis derived from poplar tree, typical for the temperate zone, can be characterized by the following parameters: total flavone and flavonol content, total flavanone and dihydroflavonol content, and total phenolic content. In routine analysis of these three main groups of bioactive substances, the rapid and low-cost spectrophotometric procedures are usually applied. Such approach is based on the failed attempts to correlate the concentration of individual active principles with the biological activity of poplar propolis. In fact, each of the identified compounds (more than 25 phenols found in poplar propolis so far) comprises different biological profiles contributing synergistically to the overall activity. Therefore, it was assumed that quantification of the active compounds within groups with the same or close chemical structures better reflects the biological activity and is more informative than quantification of individual components. For instance, propolis samples with the highest concentration of total phenols had the strongest antibacterial activity (expressed as minimum inhibitory concentration, MIC, against *S. aureus*). Based on the analysis of a large

number of samples, the following properties were suggested as characteristic of poplar propolis: flavones/flavonols (8±4) %, flavanones/dihydroflavonols (6±2) %, total phenolics (28±9) %, and MIC (211±132) µg/mL (16). Some authors have combined spectrophotometric quantification with high-performance thin layer chromatography (HPTLC) or high-performance liquid chromatography (HPLC) techniques in order to indicate the presence of some important biologically active compounds. In fact, different poplar species have a similar qualitative composition of the bud exudates, but variable quantitative composition that is reflected in the composition of the poplar propolis. Thus, caffeic acid and its esters, known allergens in European propolis, can vary from 2 to 20 % (13). One of the propolis quality control parameters, proposed by Langner and Schilcher (17), is the determination of cinnamic aldehyde as allergen with the set limit of no more than 0.05 %. Several other parameters, such as the content of waxes as biologically inert components, total ash as indicator of certain adulterations, and volatile substances as indicator of sample freshness, have been proposed for the quality control of raw propolis, while specific gravity and the percentage of ethanol have been used for the control of propolis tinctures (7). Besides the analysis of natural propolis constituents, toxic contaminants such as heavy metals and acaricide residue (insecticides used for the control of honeybee mites) should also be a subject of quality control. The purpose of propolis chemical characterisation and extraction method standardization is to connect a particular chemical type of propolis with its specific biological activity, as well as to develop a product of required quality, efficacy and safety.

#### *A case study: Standardization of Croatian propolis tincture*

Since efficacy and safety of natural remedies are based on their constant and approved quality, the chemical characterization and manufacturing standardization are postulates for their classification as medicines (18). Thus, the objective of our study was to set parameters for quality control of propolis tinctures according to the European guidelines for quality of traditional herbal medicines and state of the art in the field of propolis research, results of which are summarized in this minireview. Moreover, the method of propolis extraction was optimized with respect to time, solvent/propolis ratio, and temperature and volume fraction of ethanol, in order to obtain purified tincture with the highest concentration of extracted biologically active substances (2). For this purpose it was necessary to: (i) develop UV/VIS spectrophotometric procedures for simple and routine quantitative determination of different flavonoid groups (flavones, flavonols and flavanones) and total polyphenols (flavonoids and phenolic acids) in propolis tincture, but also to control the quality of propolis as the raw material; (ii) apply general parameters of European Pharmacopoeia prescribed for the analysis of tincture, including the development of a GC procedure for the simultaneous determination of ethanol content and detection of possible impurities (methanol and 2-propanol); and (iii) develop chromatographic procedures, HPTLC and HPLC, for

identification and quantification of individual polyphenols in propolis tincture.

According to the European quality guidelines for traditional (herbal) medicines, pharmacopoeial methods should be utilised wherever they are appropriate. Otherwise, applied analytical procedures should be validated in accordance with the International Conference on Harmonisation (ICH) guidelines (19), as has been done in this work.

#### Extraction of propolis and spectrophotometric quantification of polyphenols

The extraction of propolis was optimised through the quantitative analysis of polyphenols (flavonoids and phenolic acids) (2), the main active substances in propolis from the temperate climate zone. As chemically defined groups of constituents or marker substances, polyphenols are of interest for propolis chemical characterisation and thus its quality and efficacy evaluation. Due to extensive work with propolis extraction (70 procedures in total, combining different extraction conditions regarding ethanol fraction, temperature and time), the content of different polyphenolic groups was determined using simple and rapid spectrophotometric methods. However, it is evident from literature that the quantification of total polyphenolic and flavonoid groups reflects better the biological activity of propolis than the quantification of its individual components (16). Depending on their molecular structure, the different classes of polyphenols were quantified by three spectrophotometric procedures. The aluminium chloride method was used to determine the flavone and flavonol content; it is based on the formation of acid-stable complexes with aluminium ion between C-4 keto group and either the C-3 or C-5 hydroxyl group of the flavonoid, showing the maximum absorbance at 385–440 nm. The preliminary analysis showed that their 2,3-dihydro derivatives do not contribute to the spectrophotometric measurements based on aluminium chloride assay. These substances, flavanones, interact like ketones with 2,4-dinitrophenylhydrazine (2,4-DNPH) in acidic media, forming the orange coloured phenylhydrazones with maximum absorbance at 495 nm. Due to the presence of a double bond in position C2=C3 in the flavonoid skeleton, flavones and flavonols cannot react with 2,4-DNPH, and this characteristic distinguishes them from flavanones (20). It has also been shown that phenolic acids do not interfere with the above described procedures. Since they have a phenolic nucleus, phenolic acids were evaluated together with other polyphenols using the Folin-Ciocalteu method. This method is based on an oxidation-reduction reaction in alkaline conditions, where the phenolate ion is oxidized, while  $\text{Mo}^{6+}/\text{W}^{6+}$  complex ion from Folin-Ciocalteu reagent is reduced, turning the colour of the solution blue (7). Described methods were optimised with respect to the selection of absorbance wavelength ( $\lambda_{\text{max}}$ ) and marker substances (representatives of polyphenolic subgroups), using model mixtures of flavonoids and phenolic acids that were chosen based on the available data on Croatian propolis constituents (21,22). Validation parameters such as linearity, accuracy, repeatability, intermediate precision, stability and selectivity (19) of the three assayed spectrophotometric procedures were further examined and the obtained results

satisfied the acceptance criteria for natural drug products. Moreover, the most suitable extraction conditions were determined through accuracy testing, using recovery factors of the marker substances (galangin, pinocembrin and caffeic acid) from the real sample of propolis. As a traditional product on the market, the propolis tincture (water-alcohol extract) (23) was the object of this work and thus the extraction solvent used in the experiments was ethanol at various volume fractions (50 to 96 %). As seen in literature, among propolis preparations, the ethanol extract is the subject of intense pharmacological and chemical studies. The water extract has also been studied, but in comparison with ethanol extract, it expressed weaker antioxidant and antimicrobial activities due to lower concentration of extracted flavonoids (11). In fact, water extracts mainly contain phenolic acids as polar substances and thus are easily soluble in water. Other recorded solvents for propolis extraction (methanol, hexane, acetone, chloroform, ethyl acetate) were used only for analytical purposes and not for the development of a product with therapeutic applications (1, 24). Satisfying recovery of the chosen marker substances (96–101 % by volume) was obtained by extraction with 80 % (by volume) ethanol at room temperature ( $(21 \pm 2)$  °C) for 1 h. Authors who studied biological properties of European propolis depending on its chemical composition also concluded that the highest concentration of flavonoids was extracted with 80 % (by volume) ethanol (11). Among the commercially available propolis products tested for antimicrobial activity, preparations with the total flavonoid content above 1 % showed activity against the Gram-positive bacteria *Pseudomonas aeruginosa* and the yeast-like fungus *Candida albicans* (23). This further confirms a good correlation between the biological activity of propolis and the polyphenolic content. Since propolis is a sticky and nonhomogeneous material, for the purpose of this study the raw samples had been frozen and powdered prior to extraction. Homogenization of propolis and the use of magnetic stirrer shortened the time needed for an effective extraction in comparison with the time-consuming traditional maceration.

The robustness of spectrophotometric methods was evaluated through the stability of analytical solutions. Based on the stability results, it is recommended to carry out aluminium chloride and 2,4-DNP assays within 2 h after preparing the test solutions because of the considerable changes in the initial flavonoid concentration. The mechanisms of these changes during storage for more than 2 h were not studied in the scope of the present work.

Extracts of propolis from different Croatian regions, as well as their mixture, were prepared using the optimized extraction procedure in the mass concentration of 5 mg/mL, and then analysed by the validated spectrophotometric procedures. The obtained contents of flavones/flavonols and flavanones were added up to estimate the total content of flavonoids. Due to specific vegetation of the collection sites, the results showed expected differences in the polyphenolic content (in %): flavones and flavonols 0.53–8.24, flavanones 5.08–11.24, total flavonoids 5.81–18.28 and total polyphenols 12.72–31.72, requiring the complete chemical characterisation by chromatographic techniques. Similar to our results are

those of another study on Croatian propolis composition (in %): flavones and flavonols 2–2.3, flavanones 4–14 and total flavonoids 5–26 (25). Compared to Croatian propolis, samples from the Czech Republic and Slovakia contained higher average amounts of flavones and flavonols (5.88–9.09 %) than of flavanones (3.63–6.24 %) (26). A content of total polyphenols in Uruguayan propolis (13.10–31.20 %) is similar to our results, while slightly higher content was determined in Chinese propolis (18.80–33.10 %) (24). Apart from propolis origin, the polyphenolic content variability may also be contributed to the different extraction procedures used, emphasizing the need for extraction method standardization.

In order to obtain the optimal drug extract ratio (DER) and saturated tinctures, it is also important to adjust the amount of extraction solvent. Therefore, the extraction saturation test was performed by increasing the amount of crude propolis extracted in the same volume of solvent (5–400 mg/mL). Saturation point was assigned to the concentration of 100 mg/mL, so the chosen DER was 1:10, and thus the preparation of propolis tincture was defined. Subjected to further processing, propolis tincture can be altered into technologically more complex preparations such as non-alcoholic solution for paediatric use. Therefore, the propolis tincture was also viewed as an intermediate product for which quality control has to be done as required in quality documentation for medicines (27).

A previously described research (2) confirmed the suitability of validated spectrophotometric procedures for routine quality control of propolis, both as the starting material and in the form of tincture, regarding the total flavonoid and polyphenolic content.

#### Tests prescribed for tinctures by European Pharmacopoeia

According to the European Pharmacopoeia quality guidelines, general pharmacopoeial tests should be included in the final quality specification of a medicinal product. Quality specification is legally binding quality standard that is proposed and justified by the drug manufacturer and approved by regulatory authorities. It establishes the set of criteria to which a medicinal product should conform to be considered acceptable for its intended use, ensuring the product quality and consistency (28).

Tinctures of Croatian propolis were subjected to the analysis of the following pharmacopoeial parameters: relative density ( $d_{20}^{20}$ ), content of ethanol and its possible impurities – methanol and 2-propanol (% by volume), and dry residue of extracts (% by mass) (23). It was observed that the relative density values increased along with the increase in dry residue of the investigated tinctures. For instance, the lowest values were determined in propolis tincture from South Dalmatian islands ( $d_{20}^{20}=0.8688$  and dry residue of 4.40 %), while the highest values were obtained for propolis tincture from the area around Sisak ( $d_{20}^{20}=0.8841$  and dry residue of 7.62 %). A good correlation between total polyphenolic content and dry residues of propolis tinctures ( $R=0.8262$ ) was also observed, and when the sample from Sisak with the average polyphenolic content and the highest dry residue was excluded, this correlation was even more significant

( $R=0.9716$ ). Further research revealed a contamination of the sample from Sisak with lead. Thus, the dry residue of the tincture could be linked to the content of active compounds extracted, but also to the presence of unwanted foreign matter in the raw sample. Determination of relative density may be an indirect estimate of water content in tinctures and moisture in raw samples, showing the adequacy of their storage and processing conditions.

The ethanol content is a useful quality parameter of which the volume percentage in the tincture should be declared on the product package and on the label due to safety reasons for vulnerable patients with liver damage and epilepsy. Methanol and 2-propanol are organic solvents that originate from the ethanol production. The detection of these residual solvents is essential because of their toxic potential, and their content also indicates the quality of the ethanol used for extraction. European Pharmacopoeia describes a distillation method with pycnometer or hydrometer for determination of ethanol content (29), while gas chromatography is specified for testing methanol and 2-propanol (30). In order to simplify the quality control of propolis tincture, a GC method for the simultaneous determination of ethanol, methanol and 2-propanol content was developed and validated (2). To avoid false results, the acetone of gas chromatographic grade was used as a dilution agent instead of analytical-grade acetone, which contains certain percentage of methanol. According to the United States Pharmacopoeia (USP) (31), herbal extracts should contain between 90 and 110 % of ethanol. Since the extraction solvent used was 80 % (by volume) ethanol, the content of ethanol determined in propolis tinctures (approx. 76 % by volume) was within the obligatory range (72–88 % by volume). Contents of methanol and 2-propanol were below detection limits, therefore satisfying the European Pharmacopoeia requirements (<0.05 % by volume) (23).

#### Chromatographic analysis of polyphenolic compounds in propolis tinctures

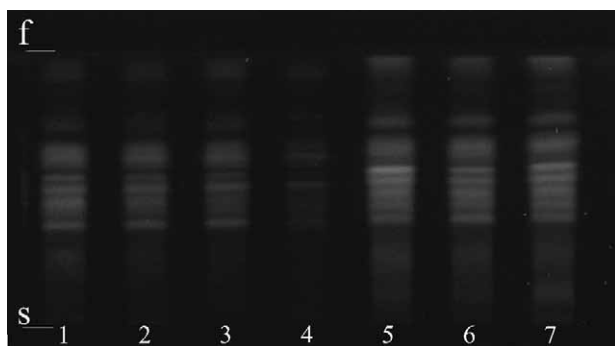
The complete chemical characterisation of propolis requires the use of various separation techniques that allow identification and quantification of individual compounds. HPTLC and HPLC are the most popular methods for routine analysis of natural compounds, according to the European Pharmacopoeia on herbal drugs/preparations.

The thin layer chromatography (TLC) is still generally regarded as the basic and simple tool for the identification of natural compounds, and is used to provide the first characteristic fingerprints of a sample. Furthermore, a modern instrumental HPTLC is widely applied in quantitative analysis. Although a two-dimensional (2D) TLC method was shown to be very useful for separation and quantitative determination of polyphenolic compounds in Croatian propolis samples (22), it also proved to be a time-consuming, expensive and complex procedure. For this reason we have established a new, simple, selective, and sensitive routine HPTLC method for the identification and quantification of three phenolic acids (caffeic, *p*-coumaric and isoferulic acids) and four flavonoids (chrysin, tectochrysin, pinocembrin and pinocembrin-7-methyl ether) in propolis tinctures (32). The com-

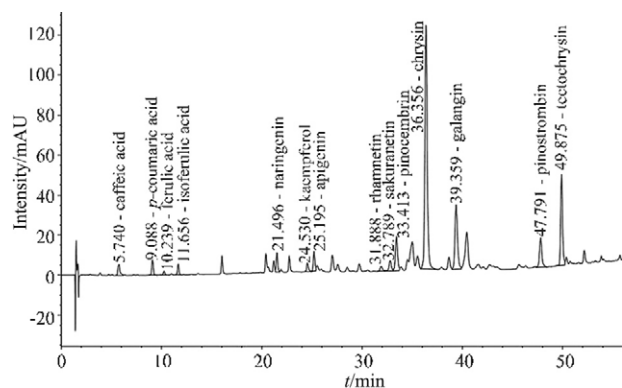
bination of chloroform as hydrophobic organic solvent with more polar methanol and formic acid was selected as the most appropriate mobile phase for the separation of the tested compounds. Chromatographic analysis was performed on glass-backed silica gel F<sub>254s</sub> HPTLC plates (Merck, Darmstadt, Germany). The advantage of the used stationary phase in comparison with conventional TLC plate is in the smaller particles ( $\leq 10 \mu\text{m}$ ), narrower particle size distribution of the sorbent and thinner sorbent layer ( $\leq 150 \mu\text{m}$ ). These provide application of smaller sample volume and larger number of samples, better resolution as well as reduced time of plate developing ( $\leq 10 \text{ cm}$  of migration distance). Unwanted effects of the so-called secondary chromatography, which leads to non-homogeneous vertical distribution of compounds inside the sorbent and thus to low reproducibility, are also reduced. The optimised HPTLC procedure was validated according to Nyiredy's (33) recommendations for validation of planar chromatographic procedures. Its analytical performance (linearity, selectivity, precision, accuracy and robustness) fulfilled the acceptance criteria established for TLC methods. Fingerprint analysis of the analysed propolis samples is shown in Fig. 1 (23), recorded with Canon (Tokyo, Japan) digital camera (using CAMAG DiggiStore 2 (Muttentz, Switzerland) documentation system). As propolis samples were collected from various regions of Croatia and its composition is highly dependent on local flora, the results showed expected differences between the types and amounts of polyphenolic compounds in the analysed tinctures. Among the tested polyphenols, caffeic acid was present in all propolis tinctures, while chrysin was the most abundant flavonoid. The tincture obtained by blending propolis of different origin contained all polyphenolic compounds studied, so it can be assumed that the quality of propolis tincture can be improved by mixing different propolis samples in order to reduce local or seasonal variations. Blending different propolis samples could also provide quantification of propolis tincture to a defined range of active markers (total phenolic acids and total flavonoids) that should be

established based on the information from manufacturing batches of tincture (28). The analysed tincture of a mixture of the Croatian propolis samples contained 0.94 mg/mL of total phenolic acids and 2.42 mg/mL of total flavonoids, with the highest content of biologically active pinocembrin (34).

Optimisation of HPLC procedure has allowed us to identify and determine the content of four phenolic acids (caffeic, *p*-coumaric, ferulic and isoferulic acids) and ten flavonoids (naringenin, kaempferol, apigenin, rhamnetin, sakuranetin, galangin, chrysin, tectochrysin, pinocembrin and pinocembrin-7-methyl ether) in propolis tinctures. For this purpose we used commonly applied reversed phase HPLC (RP-HPLC) method with a non-polar stationary phase (column packed with the octadecyl-bound silica gel) and an aqueous, moderately polar mobile phase (mixture of water and methanol with the addition of acetic acid which acts as an ion pairing agent preventing the ionization of free hydroxyl groups). To improve the separation of complex mixture such as propolis, a gradient elution with various mobile phase compositions (from solvent A consisting mainly of water to solvent B consisting mainly of hydrophobic methanol) was utilized. Under the described separation conditions, more hydrophilic compounds were eluted first. Thus, phenolic acids had the shortest retention time ( $t_R$ ). Besides the UV/VIS spectra, the retention time is considered a reasonably unique identifying characteristic of a given analyte. Based on validation criteria (18), the proposed HPLC procedure was found to be appropriate for the intended analytical purpose. Despite the differences in polyphenolic composition depending on propolis origin, all analysed tinctures contain flavones/flavonols (kaempferol, apigenin, rhamnetin, galangin, chrysin, tectochrysin), flavanones (naringenin, sakuranetin, pinocembrin, pinocembrin-7-methyl ether) and phenolic acids (caffeic, *p*-coumaric, ferulic and isoferulic acids). Therefore, the chemical characteristics of Croatian poplar propolis was confirmed. The highest concentrations of *p*-coumaric acid (0.31–3.39 mg/mL) and chrysin (1.27–10.33 mg/mL) were detected. Comparing the composition of different propolis samples from temperate climate zones, we observed that the major flavonoids are chrysin in Italian propolis (14), pinocembrin-7-methyl ether in Canadian propolis (35), galangin in German propolis, and pinocembrin in Austrian, French (36), Bulgarian (34) and New Zealand samples (37). Predominant phenolic acids in European propolis are caffeic (Italy) (14) and *p*-coumaric acid (Germany, France and Austria) (36). The poplar propolis is also characterised by the presence of phenolic acid esters (like phenylethyl caffeate, benzyl ferulate, *etc.*), which were not detected in our analysed tinctures. HPLC analysis also implied that regional variations in propolis composition (like absence of rhamnetin in some tested samples) can be modified by mixing different propolis samples. A representative chromatogram obtained from HPLC analysis of polyphenols in a mixture of Croatian propolis samples is shown in Fig. 2 (25). Within biologically active polyphenolic groups, the tincture of mixed Croatian propolis contained the highest level of caffeic acid (0.45 mg/mL), chrysin (6.39 mg/mL) and pinocembrin (1.90 mg/mL). The content of total phenolic acids (1.13 mg/mL) is similar to that determined by



**Fig. 1.** Results from fingerprint analysis of the investigated propolis tinctures – fluorescence image obtained by excitation at 366 nm after spraying with 1 % ethanolic  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  solution. 1: propolis samples collected from area around Čisla (Omiš), 2: propolis samples collected from area around Labin, 3: propolis samples collected from areas around Sisak, 4: propolis samples collected from area around Veliki Zdenci, 5: propolis samples collected from Pelješac, 6: mixture of Croatian propolis samples, 7: propolis samples collected from areas around Metković (s= start line, f=solvent front) (23)



**Fig. 2.** HPLC chromatogram of the tincture of mixed Croatian propolis samples analyzed at  $\lambda=270$  nm, obtained by binary gradient elution (solvent A: water/ acetic acid/methanol=88:2:10 by volume, and solvent B: methanol/acetic acid/water=90:2:8 by volume) and using column XBridge C18 (150×3.0 mm i.d., particle size 3.5  $\mu$ m; Waters, Dublin, Ireland) (25)

the HPTLC method (0.94 mg/mL), while the content of total flavonoids (16.09 mg/mL) is slightly higher than that determined using spectrophotometric method (12 mg/mL). Almost equal results justify the use of rapid and low-cost spectrophotometric and HPTLC procedures in routine quality control of propolis tincture. However, HPLC analysis should be performed if more reliable information regarding the composition of polyphenols is required.

## Wine as a Functional Food

Wine, especially red wine, contains a wide range of polyphenols that include phenolic acid, trihydroxystilbene resveratrol, flavonols (*e.g.* quercetin and myricetin), flavan-3-ols (*e.g.* catechin and epicatechin), as well as polymers of the latter, defined as procyanidins and anthocyanins. These compounds are characteristic for quality attributes of wine, and they contribute to its colour and sensory properties, such as flavour and astringency (38). Also, they manifest a wide range of beneficial health effects including antioxidative, antiallergic, anti-inflammatory, antidiabetic, hepato- and gastro-protective, antiviral, and antineoplastic activities (39). A great deal of studies have shown the antioxidative properties of these compounds in protection against arteriosclerosis and coronary heart disease because they inhibit the oxidation of human LDL and protect against the development of atheroma (40). Since beneficial effects of wine are mostly related to the biological activities of polyphenols, the following text will focus on their content in wine.

### Polyphenols in wine

Polyphenols from grape skin, seeds, and flesh are extracted into wine during the process of vinification. The simplest compounds are hydroxycinnamic acids (caffeic, ferulic and *p*-coumaric acids) and hydroxybenzoic acids (gallic, syringic and vanillic acids), and their esters, while more complex compounds are flavonoids, which are present in the grape mainly in the monoglycoside form, with the sugar residue linked to the hydroxyl group in posi-

tion C-3 of the C ring. The glycoside flavonols kaempferol, quercetin and myricetin, which form copigments with anthocyanins in red wines, together with the products of tannin oxidation, are mainly responsible for the colour of white grapes and wines. Anthocyanins contain the benzopyrylium ion as a base molecule in their skeleton, which is responsible for the colour of red berry varieties and red wines. They are present in the grape as mono- or diglycosides, depending on variety, with the second glucose molecule linked to the C-5 hydroxyl group. The flavanols (+)-catechin, (+)-gallocatechin, (-)-epicatechin and (-)-epigallocatechin are present in the grape as monomers, while condensed procyanidins, proanthocyanidins and tannins consist of flavonol units (41–43).

### Factors influencing polyphenolic content of Croatian wines

The content and profile of polyphenols in wine are influenced by an intrinsic factor such as grape variety, and extrinsic factors such as atmospheric conditions, process of viticulture and techniques employed during vinification. Because of their unique varietal (genetic) diversity, grapes may vary largely in the polyphenolic composition. Therefore, the examination of polyphenolic composition using different techniques, such as HPLC or capillary electrophoresis (CE), is an indispensable element in choosing (and/or blending) the appropriate grape varieties and selecting the technological applications, which enables the production of high quality wines. By means of multivariate data analysis, such as principle component analysis, cluster analysis and artificial neural networking, it is possible to obtain the classification of grape varieties based on their different polyphenolic content, *i.e.* fingerprints (44,45). The results of the study of polyphenolic profile of grape skin extracts of fourteen *Vitis vinifera* varieties grown in Dalmatia (Croatia) confirmed the varietal dependence of the content of total polyphenols and individual polyphenols. Skins of red cultivars Rudežuša and Trnjak and of white cultivars Debit, Zlatarica and Kujundžuša have the highest concentration of individual polyphenolic subgroups (46). In the study of free resveratrol monomers in varietal red and white wines from Dalmatia (Croatia), the highest concentrations were found in red wine produced from grape cultivar Plavac mali, while among white wines, the highest concentrations were detected in wine Zlatarica (47).

The accumulation of flavonoids in grapes is enhanced by the increase of light exposure, especially ultraviolet B rays. Consequently, grapes exposed to increased daylight are capable of biosynthesizing more flavonols, so high total flavonol levels in red wines have been associated with the grapes grown in a sunnier microclimate. Thus, wines made from highly and moderately exposed clusters at higher positions have higher total anthocyanin levels than those from shaded clusters (48,49). Therefore, flavonol content in wines from Croatia is associated with the geographical origin, since higher levels of polyphenols were detected in wines from the coastal region, with Mediterranean climate, as opposed to other parts of Croatia with continental climate (50,51).

The polyphenolic content of grapes can also be modified by environmental conditions and viticultural practices. The use of fertiliser(s) usually results in increased yield, but excessive or unbalanced fertilization can have negative effects on polyphenolic content. Moderate nitrogen supply before blooming enhances the synthesis of polyphenols in grapes. Nevertheless, an increase in the production capacity of the plant is often associated with the increase of berry size, so an increase in the pulp/skin ratio causes dilution of the anthocyanins and tannins in the must. Also, adequate potassium fertilization helps to increase both the colour and the polyphenolic content of berries (50,52). Recent study of phenolic content and antioxidant activity of Croatian wines, deriving from organically and conventionally grown grapes, has shown that the antioxidant activity and concentration of all studied hydroxybenzoic acids and flavonols are higher in organic wines (53).

Grape quality is also an important determinant of the eventual phenolic content of a wine. Disease can reduce berry quality, leading to a lower polyphenolic content of harvested grapes. A high-quality grape has a higher berry surface to volume ratio compared to the lower-quality grape. Because of the small size of berries of high-quality grapes, a much greater volume of these grapes is required in order to make the same volume of wine as with the lower-quality grapes. Wines made from high-quality grapes have a higher content of skin-derived phenols than those made from lower-quality grapes. Also, smaller harvest will also result in increased phenolic concentrations within grapes, in comparison with an excessively large harvest (54,55).

Winemaking techniques (time of maceration, destemming of grape clusters, addition of a supplementary quantity of seeds to musts, fermentation conditions, *etc.*) greatly influence the polyphenolic content. The experiments show that there is a progressive increase in the content of catechins and proanthocyanidins, but a decrease of total and some individual anthocyanins with the increased length of maceration time. Similarly, the presence of stems during fermentation gives wines with a higher content of catechins and proanthocyanidins. Significant differences have been found in the composition of various phenolic compounds (catechins, oligomeric and polymeric proanthocyanidins, anthocyanins) and volatile compounds (alcohols and esters) among red wines made by different winemaking technologies. The wines obtained by carbonic maceration had less intense colour, lower concentration of polyphenols, but higher concentration of volatile compounds than the wines obtained by conventional alcoholic fermentation (56,57). Results of the study published by Piljac *et al.* (58) revealed a great difference in total phenolic content of red wine produced in Croatia and of wine produced in New Zealand. The difference was explained by the traditional processing of grapes in Croatia, with prolonged maceration time.

The polyphenolic profile of red wines is fundamentally different from that of white wines due to differences in the composition of red and white grapes, as well as the implementation of different vinification technologies. Traditional white wine vinification usually precludes contact of must with grape pomace and, as a con-

sequence, extraction of flavonols that are mainly located in the skin is very limited. Post-fermentation treatments such as fining were found to cause significant reduction in both flavonol glycoside and aglycone content of Sherry wines (59).

Factors that may affect the polyphenolic composition are also associated with ageing and storage conditions. Ageing in oak wood allows the extraction of a series of benzoic and cinnamic compounds (vanillin, vanillic acid, syringaldehyde, syringic acid, coniferaldehyde, sinapaldehyde), gallic acid, ellagic acid and cummarines (scopoletine, umbelliferone) into the wine, so the wines produced by fermentation and maturation in oak barrels have different flavour characteristics than those that have undergone barrel maturation after fermentation in stainless steel tanks (60).

#### *Analysis of polyphenols from Croatian wines*

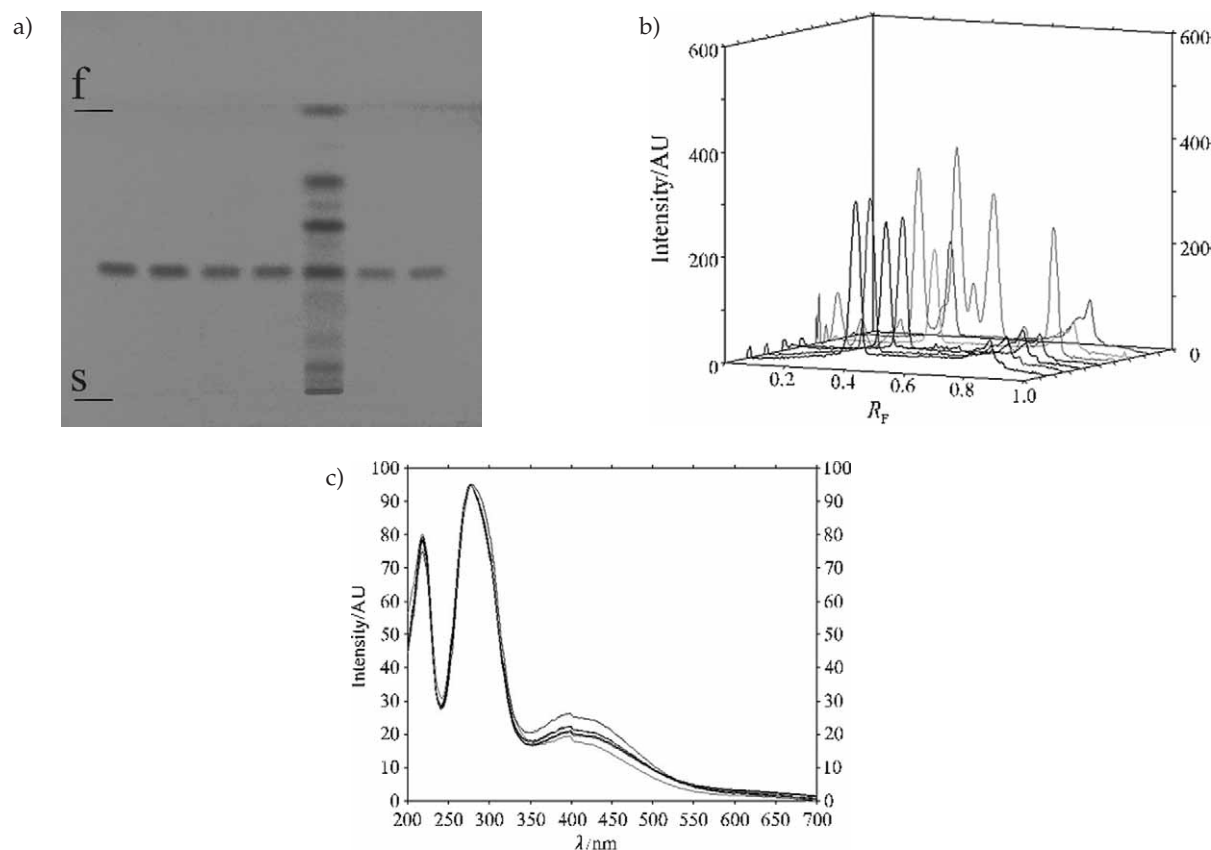
Numerous studies have been performed in the attempt to analyse polyphenols in both propolis and wine by means of various methods including TLC, HPLC, GC, and CE.

TLC on silica gel sheets is a good method for relatively fast separation and identification of the phenolic compounds present in wine samples. Qualitative analysis of the proanthocyanidin fractions of wine can be obtained by solid-phase extraction and separation on the basis of their degree of polymerization (61). Information theory and clustering methods have been used to select and evaluate the efficiency of 11 used mobile phases for the determination of polyphenols in Croatian wines. The application of the most appropriate mobile phase (benzene/ethyl acetate/formic acid=60:30:10 by volume) allowed the identification of several polyphenolic compounds (62). Densitometric quantitative analysis of polyphenols in wine extracts is usually performed by scanning the TLC plates with UV light at wavelengths of 350–365 or 250–260 nm. TLC quantification of polyphenols in wine was performed using CAMAG system. The substances were identified on the basis of retention values and UV spectra (Fig. 3) (63).

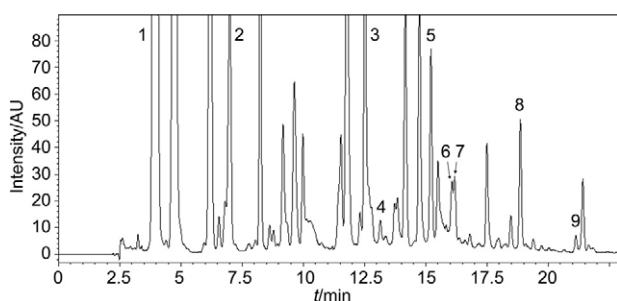
HPLC currently represents the most popular technique for the analysis of polyphenols in wine. For this purpose, a RP-HPLC method that uses gradient elution with binary elution system is usually employed. Routine detection is based on the measurement of UV/VIS absorption with a diode array detector (DAD) (Fig. 4) (45, 50). Enhancing selectivity and sensitivity for the determination of certain polyphenols requires the application of different detection techniques, such as fluorimetry (64), electrochemistry (65), chemiluminescence, and/or mass spectrometry coupled with ionization techniques: electrospray ionization (ESI) (66), matrix-assisted laser desorption/ionization (MALDI) (67), and atmospheric pressure chemical ionization (APCI) (68).

Several GC/MS analytical methods have been developed to characterize and quantify phenolic compounds in wine which require derivatization of volatile compounds and mass-spectrometric detection in the selective ion-monitoring mode (GC/MS-SIM) (69,70). CE is rapidly developing analytical tool successfully used for analysis of polyphenols in red and white wines with an





**Fig. 3.** Densitometric quantitative analysis of polyphenols from Croatian red wine Postup: a) photograph of the chromatographic plate coated with silica gel (60 F<sub>254</sub>), mobile phase: benzene/ethyl acetate/formic acid=60:30:10 by volume, recorded at  $\lambda=254$  nm during the determination of gallic acid in a sample of red wine Postup (s=start line, f=solvent front); b) three-dimensional diagram (densitogram) obtained by scanning the same chromatographic plate using a densitometer; c) spectrum of marked peaks obtained using CAMAG densitometer (63)



**Fig. 4.** HPLC chromatogram of a Croatian red wine Dingač extract recorded at 280 nm. Gradient elution with two solvents was used: solvent A: phosphoric acid (pH=3.0)/methanol (90:10 by volume), and solvent B: phosphoric acid (pH=3.0)/methanol (10:90 by volume). Assigned polyphenols: 1) gallic acid, 2) catechin, 3) *p*-coumaric acid, 4) ferulic acid, 5) *trans*-resveratrol, 6) myricetin, 7) quercitrin, 8) quercetin, 9) kaempferol (50)

opportune sample preconcentration step. Micellar electrokinetic capillary chromatography (MECC) has extended the utility of capillary electrophoresis to the separation of neutral analytes under the influence of an electric field. Fractionation of monomeric and polymeric pigments of higher molecular mass by gel permeation chromatography (GPC) improved the analysis of these compounds by CE (44,71).

## Conclusion and Future Prospects

In the presented study, the preparation of propolis tincture was standardized, forming the groundwork for its quality control. For that purpose, several analytical procedures for which suitability was proved through validation experiments were employed. The proposed quality specification for propolis tincture is given in Table 1. Since final specification can only be established based on the information on manufacturing batches of tincture, we proposed the requirements that are based on our results of representative sample analysis, *i.e.* the mixture of Croatian propolis samples. Where it is applicable, requirements prescribed in literature are used as shown in the third column of Table 1, in brackets. For defining the limits of polyphenolic content, it is also advisable to conduct biological assay, so the final standardization of propolis tincture is based on its efficacy. As demonstrated in Table 1, the polyphenolic concentration determined in propolis tincture differs depending on the analytical procedure used as a result of different method selectivity. The spectrophotometric and HPTLC procedures showed to be appropriate for rapid and routine quality control of propolis tinctures, while HPLC is recommended for obtaining more reliable data on flavonoid and phenolic acid composition. Besides identification and quantification of biologically active substances, the proposed quality speci-

Table 1. Parameters for quality control assessment of propolis tincture

Test	Analytical procedure	Results for a mixture of Croatian propolis
appearance	organoleptic testing	brown liquid of characteristic aromatic odour; a slight sediment may form on standing
relative density ( $d_{20}$ )	Ph. Eur. 2.2.5	0.8803
dry residue ( $w/\%$ )	Ph. Eur. 2.8.16	6.56
identification and assay of phenolics ( $\gamma/(\text{mg/mL})$ ):		
flavones/flavonols ( $\lambda=415$ nm)	UV/VIS	4.05
flavanones ( $\lambda=495$ nm)		7.95
total phenols ( $\lambda=760$ nm)		24.94
total flavonoids	HPTLC (4)* HPLC (10)*	2.42 16.09
total phenolic acids	HPTLC (3)* HPLC (4)*	0.94 1.13
$\phi(\text{ethanol})/\%$	GC	75.57 (USP: 72–88 %)
methanol and 2-propanol	GC	<DL (Ph.Eur.: $\leq 0.05$ % by volume)
microbiological quality	Ph. Eur. 2.6.12, 2.6.13	Ph. Eur. 5.1.4., category 3B
waxes in raw propolis ( $w/\%$ )	gravimetric analysis	8.75

\*the number in brackets corresponds to the number of analyzed phenolic acids and flavonoids  
Ph. Eur.=European Pharmacopoeia, USP=US Pharmacopoeia

fication also includes general pharmacopoeial parameters specified for tinctures. Moreover, the new gas chromatographic procedure was proposed for the simultaneous determination of ethanol content and detection of its impurities (methanol and 2-propanol). The quality specification for propolis tincture should also prescribe microbiological purity examination. Since propolis tincture displays antimicrobial activity itself, microbiological quality testing is only an indicator of good manufacturing practice compliance.

The presented studies about Croatian wines suggest that polyphenolic profile of wine can be used for chemotaxonomy of wines of various grape varieties and geographical origins. Regular moderate wine consumption has been associated with several health benefits. These findings support the growing body of scientific research indicating that moderate consumption of alcoholic beverages is associated with lower levels of coronary heart disease, as well as with better health and greater longevity. These effects are thought to be accomplished by several compounds that are found in wine, particularly antioxidants such as polyphenols. Although a lot more is known about the phenolic components of red wine, we still do not know whether the components have individual functions and which are the most important.

Also, very little research has been done on the metabolism and biological activities of phenolics at cellular, molecular and biochemical levels. Further research is therefore required, especially *in vivo* supplementation studies with pure flavonoids and mixtures of flavonoid compounds to identify the biological activities of their metabolites.

Any functional food has to fulfill three main goals: known and uniform composition, health benefits, and safety. All these are achieved by setting good standards for chemical analysis, *e.g.* certificate of analysis for propolis (Table 1). Nutraceuticals that have pharmacological

activity, from which health benefits could be claimed, should be well defined and labelled on the sample. Above all, all possible contaminants, such as toxic metals, pesticides and radionuclides, should be controlled to rationalize safety of usage.

#### Acknowledgements

The authors would like thank to the Croatian Ministry of Science, Education and Sports for the financial support through the project No. 006-0061117-1237 (M. M.-Š.).

#### References

1. A.M. Gómez-Caravaca, M. Gómez-Romero, D. Arráez-Román, A. Segura-Carretero, A. Fernández-Gutiérrez, Advances in the analysis of phenolic compounds in products derived from bees, *J. Pharm. Biomed. Anal.* 41 (2006) 1220–1234.
2. J. Cvek, M. Medić-Šarić, I. Jasprica, S. Zubčić, D. Vitali, A. Mornar *et al.*, Optimisation of an extraction procedure and chemical characterisation of Croatian propolis tincture, *Phytochem. Anal.* 18 (2007) 451–459.
3. B. Trusheva, D. Trunkova, V. Bankova, Different extraction methods of biologically active components from propolis: A preliminary study, *Chem. Central J.* 1 (2007) 13–16.
4. R. Krell, Value-added products from beekeeping, *FAO Agricultural Services Bulletin, No. 124*, Food and Agriculture Organization of the United Nations, Rome, Italy (1996).
5. R.C. Peña, Propolis standardization: A chemical and biological review, *Ciênc. Inv. Agr.* 35 (2008) 11–20.
6. G.A. Burdock, Review of the biological properties and toxicity of bee propolis (propolis), *Food Chem. Toxicol.* 36 (1998) 347–363.
7. I.B.S. Cunha, M.L.T. Rodrigues, E.C. Meurer, V.S. Bankova, M.C. Marcucci, M.N. Eberlin, A.C.H.F. Sawaya, Effect of the maceration time on chemical composition of extracts of Brazilian propolis, *J. Apic. Res.* 45 (2006) 137–144.
8. I.B.S. Cunha, A.C.H.F. Sawaya, F.M. Caetano, M.T. Shimizu, M.C. Marcucci, F.T. Drezza *et al.*, Factors that influence

- the yield and composition of Brazilian propolis extracts, *J. Braz. Chem. Soc.* 15 (2004) 964–970.
9. R.G. Woisky, A. Salatino, Analysis of propolis: Some parameters and procedures for chemical quality control, *J. Apic. Res.* 37 (1998) 99–105.
  10. Beeswax. In: *European Pharmacopoeia, Vol. 2*, Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM), Strasbourg, France (2007) pp. 1260–1261.
  11. Y.K. Park, M. Ikegaki, Preparation of water and ethanolic extracts of propolis and evaluation of the preparations, *Biosci. Biotechnol. Biochem.* 62 (1998) 2230–2232.
  12. M. Vanhaelen, R. Vanhaelen-Fastré, Propolis I. Origin, microscopical investigations, chemical constituents and therapeutic activity, *J. Pharm. Belg.* 34 (1979) 253–259 (in French).
  13. V. Bankova, M.C. Marcucci, Standardization of propolis: Present status and perspectives, *Bee World*, 81 (2000) 182–188.
  14. P.G. Pietta, C. Gardana, A.M. Pietta, Analytical methods for quality control of propolis, *Fitoterapia (Suppl.)*, 73 (2002) 7–20.
  15. M.L. Bruschi, S.L. Franco, M.P.D. Gremião, Application of an HPLC method for analysis of propolis tinctures, *J. Liq. Chrom. Rel. Technol.* 26 (2003) 2399–2409.
  16. V. Bankova, Chemical diversity of propolis and the problem of standardization, *J. Ethnopharmacol.* 100 (2005) 114–117.
  17. V.E. Langner, H. Schilcher, Propolis. Quality and the activity of propolis and propolis-based products, *Deutsch. Apoth. Zeit.* 139 (1999) 51–64 (in German).
  18. R. Bauer, Quality criteria and standardization of phytopharmaceuticals: Can acceptable drug standards be achieved?, *Drug. Inf. J.* 32 (1998) 101–110.
  19. Validation of Analytical Procedures: Text and Methodology Q2(R1), International Conference on Harmonisation (ICH) of Technical Requirements for the Registration of Pharmaceuticals for Human Use, Geneva, Switzerland (2005).
  20. C.C. Chang, M.H. Ming, H.M. Wen, J.C. Chern, Estimation of total flavonoid content in propolis by two complementary colorimetric methods, *J. Food Drug Anal.* 10 (2002) 178–182.
  21. I. Jasprica, A. Smolčić-Bubalo, A. Mornar, M. Medić-Šarić, Investigation of the flavonoids in Croatian propolis by thin-layer chromatography, *J. Planar Chromatogr.* 17 (2004) 95–101.
  22. M. Medić-Šarić, I. Jasprica, A. Mornar, A. Smolčić-Bubalo, P. Golja, Quantitative analysis of flavonoids and phenolic acids in propolis by two-dimensional thin layer chromatography, *J. Planar Chromatogr.* 17 (2004) 459–463.
  23. J. Cvek, Chemical characterisation and standardization of propolis tincture, *PhD Thesis*, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia (2009) (in Croatian).
  24. J.S. Bonvehi, F.V. Coll, Phenolic composition of propolis from China and from South America, *Z. Naturforsch. C*, 49 (1994) 712–718.
  25. A. Smolčić-Bubalo, New methods in research of Croatian propolis, *PhD Thesis*, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia (2007) (in Croatian).
  26. M. Nagy, D. Grancai, Colorimetric determination of flavanones in propolis, *Pharmazie*, 51 (1996) 100–101.
  27. Guideline on the Use of the CTD Format in the Preparation of a Registration Application for Traditional Herbal Medicinal Products (EMA/HMPC/71049/2007), European Agency for the Evaluation of Medicinal Products, London, UK (2008).
  28. Guideline on Specifications: Test Procedures and Acceptance Criteria for Herbal Substances, Herbal Preparations and Herbal Medicinal Products/Traditional Herbal Medicinal Products (CPMP/QWP/2820/00 Rev. 1), European Agency for the Evaluation of Medicinal Products, London, UK (2006).
  29. Ethanol Content and Alcoholimetric Tables. In: *European Pharmacopoeia, Vol. 1*, Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM), Strasbourg, France (2007) pp. 281–282.
  30. Test for Methanol and 2-Propanol. In: *European Pharmacopoeia, Vol. 1*, Directorate for the Quality of Medicines & HealthCare of the Council of Europe (EDQM), Strasbourg, France (2007) pp. 282–283.
  31. Botanical Extracts – General Chapters (565), U.S. Pharmacopeia and the National Formulary, U.S. Pharmacopeial Convention, Rockville, MD, USA (2006).
  32. J. Cvek, M. Medić-Šarić, I. Jasprica, A. Mornar, High-performance-thin-layer-chromatographic method for estimation of phenolic acids and flavonoids content in Croatian propolis samples, *J. Planar Chromatogr.* 20 (2007) 429–435.
  33. S. Nyiredy: *Planar Chromatography – A Retrospective View for the Third Millennium*, Springer Scientific Publishing, Budapest, Hungary (2001) pp. 336–352.
  34. V.S. Bankova, S.S. Popov, N.L. Marekov, High-performance liquid chromatographic analysis of flavonoids from propolis, *J. Chromatogr.* 242 (1982) 135–143.
  35. C. García-Viguera, F. Ferreres, F.A. Tomás-Barberán, Study of Canadian propolis by GC-MS and HPLC, *Z. Naturforsch. C*, 48 (1993) 731–735.
  36. A.G. Hegazi, F.K.A. Abd El Hady, F.A.M. Abd Allah, Chemical composition and antimicrobial activity of European propolis, *Z. Naturforsch. C*, 55 (2000) 70–75.
  37. K.R. Markham, K.A. Mitchell, A.L. Wilkins, J.A. Daldy, Y. Lu, HPLC and GC-MS identification of the major organic constituents in New Zealand propolis, *Phytochemistry*, 42 (1996) 205–211.
  38. I. Lesschaeve, A.C. Noble, Polyphenols: Factors influencing their sensory properties and their effects on food and beverage preferences, *Am. J. Clin. Nutr. (Suppl.)*, 81 (2005) 330–335.
  39. L.H. Yao, Y.M. Jiang, J. Shi, F.A. Tomás-Barberán, N. Datta, R. Singanusong, S.S. Chen, Flavonoids in food and their health benefits, *Plant Food Hum. Nutr.* 59 (2004) 113–122.
  40. B. Fuhrman, A. Lavy, M. Aviram, Consumption of red wine with meals reduces the susceptibility of human plasma and low-density lipoprotein to lipid peroxidation, *Am. J. Clin. Nutr.* 61 (1995) 549–554.
  41. R. Flamini, Mass spectrometry in grape and wine chemistry. Part I: Polyphenols, *Mass Spectrom. Rev.* 22 (2003) 218–250.
  42. A. de Villiers, G. Vanhoenacker, P. Majek, P. Sandra, Determination of anthocyanins in wine by direct injection liquid chromatography–diode array detection–mass spectrometry and classification of wines using discriminant analysis, *J. Chromatogr. A*, 1054 (2004) 195–204.
  43. E. Haslam: *Practical Polyphenolics: From Structure to Molecular Recognition and Physiological Action*, Cambridge University Press, Cambridge, UK (1998).
  44. J. Pazourek, D. Gajdoševá, M. Spanilá, K. Farková, J. Havel, Analysis of polyphenols in wines: Correlation between total polyphenolic content and antioxidant potential from photometric measurements. Prediction of cultivars and vintage from fingerprints using artificial neural network, *J. Chromatogr. A*, 1081 (2005) 48–54.
  45. S. Kallithraka, E. Tsoutsouras, P. Tzourou, P. Lanaridis, Principal phenolic compounds in Greek red wine, *Food Chem.* 99 (2006) 784–793.

46. V. Katalinić, S.S. Možina, D. Skroza, I. Generalić, H. Abramović, M. Miloš *et al.*, Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* grown in Dalmatia (Croatia), *Food Chem.* 119 (2010) 715–723.
47. V. Katalinić, I. Ljubenković, I. Pezo, I. Generalić, O. Stričević, M. Miloš, D. Modun, M. Boban, Free resveratrol monomers in varietal red and white wines from Dalmatia (Croatia), *Period. Biol.* 110 (2008) 77–83.
48. M. McDonald, M. Hughes, J. Burns, M.E.J. Lean, D. Matthews, A. Crozier, Survey of the free and conjugated myricetin and quercetin content of red wines of different geographical origins, *J. Agric. Food Chem.* 46 (1998) 368–375.
49. N. Mateus, S. Proença, P. Ribeiro, J.M. Machado, V. De Freitas, Grape and wine polyphenolic composition of red *Vitis vinifera* varieties concerning vineyard altitude, *Ciênc. Technol. Aliment.* 3 (2001) 102–110.
50. V. Rastija, G. Srečnik, M. Medić-Šarić, Polyphenolic composition of Croatian wines with different geographical origins, *Food Chem.* 115 (2009) 54–60.
51. M. Šeruga, I. Novak, L. Jakobek, Determination of polyphenols and antioxidant activity of some red wines by differential pulse voltammetry, HPLC and spectrophotometric methods, *Food Chem.* 124 (2011) 1208–1216.
52. R. Delgado, P. Martín, M. del Álamo, M.R. González, Changes in the phenolic composition of grape berries during ripening in relation to vineyard nitrogen and potassium fertilisation rate, *J. Sci. Food Agric.* 84 (2004) 623–630.
53. I. Vinković Vrček, M. Bojić, I. Žuntar, G. Mendaš, M. Medić-Šarić, Phenol content, antioxidant activity and metal composition of Croatian wine deriving from organically and conventionally grown grapes, *Food Chem.* 124 (2011) 354–361.
54. L.A. Weston, Grape and wine tannins and phenolics – Their roles in flavor, quality and human health, *Wine Industry Workshop Proceedings*, New York State Agricultural Experiment Station Department of Food Science and Technology, New York, NY, USA (2000) pp. 6–14.
55. J. Burns, P.T. Gardner, D. Matthews, G.G. Duthie, M.E.J. Lean, A. Crozier, Extraction of phenolics and changes in antioxidant activity of red wines during vinification, *J. Agric. Food Chem.* 49 (2001) 5797–5808.
56. V. Kovač, E. Alonso, M. Bourzeix, E. Revilla, Effect of several enological practices on the content of catechins and proanthocyanidins of red wines, *J. Agric. Food Chem.* 40 (1992) 1953–1957.
57. M.I. Spranger, M.C. Clímaco, B. Sun, N. Eiriz, C. Fortunato, A. Nunes *et al.*, Differentiation of red winemaking technologies by phenolic and volatile composition, *Anal. Chim. Acta*, 513 (2004) 151–161.
58. J. Piljac, S. Martinez, T. Stipčević, Ž. Petrović, M. Metikoš-Huković, A cyclic voltammetry investigation of the phenolic content of Croatian wines, *Am. J. Enol. Vitic.* 55 (2004) 417–422.
59. D.P. Makris, S. Kallithraka, P. Kefalas, Flavonols in grapes, grape products and wines: Burden, profile and influential parameters, *J. Food Compos. Anal.* 19 (2006) 396–404.
60. M.A. Sanza, I.N. Domínguez, L.M.C. Cárcel, L.G. Gracia, Analysis for low molecular weight phenolic compounds in a red wine aged in oak chips, *Anal. Chim. Acta*, 513 (2004) 229–237.
61. B. Sun, C. Leandro, J.M.R. da Silva, I. Spranger, Separation of grape and wine proanthocyanidins according to their degree of polymerization, *J. Agric. Food Chem.* 46 (1998) 1390–1396.
62. V. Rastija, A. Mornar, I. Jasprica, G. Srečnik, M. Medić-Šarić, Analysis of phenolic components in Croatian red wine by thin-layer chromatography, *J. Planar Chromatogr.* 17 (2004) 26–31.
63. V. Rastija, M. Medić-Šarić, Chromatographic methods for the analysis of polyphenols in wines, *Kem. Ind.* 58 (2009) 121–128.
64. P. Viñas, C. López-Erroz, J.J. Marín-Hernández, M. Hernández-Córdoba, Determination of phenols in wines by liquid chromatography with photodiode array and fluorescence detection, *J. Chromatogr. A*, 871 (2000) 85–93.
65. P. Mattila, J. Astola, J. Kumpulainen, Determination of flavonoids in plant material by HPLC with diode-array and electro-array detections, *J. Agric. Food Chem.* 38 (2000) 5834–5841.
66. G. Stecher, C.W. Huck, M. Popp, G.K. Bonn, Determination of flavonoids and stilbenes in red wine and related biological products by HPLC and HPLC–ESI–MS–MS, *Fresenius J. Anal. Chem.* 371 (2001) 73–80.
67. A.C. Muñoz-Espada, K.V. Wood, B. Bordelon, B.A. Watkins, Anthocyanin quantification and radical scavenging capacity of Concord, Norton, and Marechal Foch grapes and wines, *J. Agric. Food Chem.* 52 (2004) 6779–6786.
68. M.N. Bravo, S. Silva, A.V. Coelho, L. Vilas Boas, M.R. Bronze, Analysis of phenolic compounds in Muscatel wines produced in Portugal, *Anal. Chim. Acta*, 563 (2006) 84–92.
69. C. Betés-Saura, C. Andrés-Lacueva, R.M. Lamuela-Raventós, Phenolics in white free run juices and wines from Penedès by high-performance liquid chromatography: Changes during vinification, *J. Agric. Food Chem.* 44 (1996) 3040–3046.
70. L. Minuti, R.M. Pellegrino, I. Tesei, Simple extraction method and gas chromatography–mass spectrometry in the selective ion monitoring mode for the determination of phenols in wine, *J. Chromatogr. A*, 1114 (2006) 263–268.
71. R.C. Minussi, M. Rossi, L. Bologna, L. Cordi, D. Rotilio, G.M. Pastore, N. Durán, Phenolic compounds and total antioxidant potential of commercial wines, *Food Chem.* 82 (2003) 409–416.