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Modern analytical techniques in the assessment of the authenticity of Serbian honey

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Food authenticity in a broader sense means fulfilling chemical and physical criteria prescribed by the proposed legislation. In the case of honey authenticity, two aspects are of major concern: the manufacturing process and the labelling of final products in terms of their geographical and botanical origin. A reliable assessment of honey authenticity has been a long-term preoccupation of chemists-analysts and it usually involves the use of several criteria and chemical markers, as well as a combination of analytical and statistical (chemometric) methods. This paper provides an overview of different criteria and modern methods for the assessment of honey authenticity in the case of a statistically significant number of authentic honey samples of several botanical types from various regions of Serbia.

KEY WORDS: *analytical methods; botanical origin of honey; chemical characterisation; geographical origin of honey; multifloral honey; unifloral honey*

According to the Merriam-Webster dictionary, the meaning of the word "authentic" is: *i*) real or genuine *ii*) not copied or false, *iii*) true and accurate, and *iv*) made to be or look just like an original, while "to authenticate" means *i*) to prove that something is real, true, or genuine, and *ii*) to prove that something is authentic (1). Although there is no official definition of "food authenticity", food is authentic if it: *i*) complies with legislation, *ii*) has the necessary composition for a legal name, *iii*) matches the description on the label, and *iv*) is not economically adulterated by substitution of its ingredients with similar but cheaper ones (2). Therefore, to test for the authentication implies confirming all requirements regarding product description or detection of fraudulent statements according to the proposed legal regulations have been fulfilled (3-5).

Verifying the description of food in terms of its composition, processing or origin is a challenging task for chemists in food analysis, commonly addressed using sophisticated analytical methods and multivariate data analysis (6). The classic authenticity assessment of food is usually based on finding a specific marker or markers, *i.e.* one or two food ingredients or their specific ratio, which are indicative of certain property of the product (3, 5). This challenging task could be solved by analysing the statistically significant number of authentic samples of a given foodstuff with respect to both natural variation of the investigated criterion and analytical uncertainty. Commonly, food authentication procedure is based on determination of parameters which do not undergo too many alterations during food processing (7-9).

Different analytical techniques may be used for the estimation of food authenticity, such as various chromatographic methods (gas chromatography – GC; (high-performance) liquid chromatography – (HP)LC; (high-performance) thin-layer chromatography – (HP) TLC); electrophoresis; spectroscopic techniques (ultraviolet-visible spectrophotometry; infrared (IR), Raman, fluorescence and nuclear magnetic resonance (NMR) spectroscopy); stable isotope analysis (stable isotope ratio mass spectrometry – IRMS, site-specific natural isotope fractionation-NMR – SNIF-NMR); enzymatic, immunological and DNA-based methods as well as metabolomics (6, 10, 11).

Authenticity of honey

In accordance with the European Union Directive (2001/110/EC) and Codex Alimentarius (Codex STAN 12-1981), honey is defined as "the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants, or from secretions of living parts of plants, or excretions of plant sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature" (12, 13). Chemically, honey is a concentrated aqueous solution of different carbohydrates (fructose, glucose, maltose, sucrose and other oligo- and polysaccharides (14); it also contains a very complex mixture of amino acids, proteins, organic acids,

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minerals, volatile compounds, various polyphenolics, vitamins, pigments, waxes, pollen grains, enzymes, and other phytochemicals. Honey reportedly contains a few hundred various substances (15). The composition and properties of this complex mixture depend on different factors such as bee species, nectar-providing plant species, geographical area, season, mode of storage, and even harvest technology and conditions (16).

Since ancient times, honey has been used as both a nutrient and a medicine (17). This natural sweetener is a very important source of carbohydrates but it also contains a significant amount of various phenolic substances with antioxidant and health-promoting effects such as reduction of heart diseases, cancer, immune system decline, and control of different inflammatory processes (18). The use of honey and other bee products for the treatment of various human diseases is a subject of an alternative medicine branch named apitherapy.

The chemical composition and, hence, the properties of honey depend largely on its floral source which is, again, closely associated to the geographical origin of honey (16). Since accumulation of phytochemicals depends on soil characteristics and climatic conditions such as sunlight and moisture, the chemical composition of honeys may be quite different even with identical botanical origin (19, 20).

Generally, honeys are classified as unifloral or monofloral, and multifloral or polyfloral. Unifloral honey predominantly originates from the nectar of one plant species with minor contributions from other floral origins, while multifloral honey has several botanical sources, without the predominance of any plant.

According to the European Council Directive 2001/110/EC concerning honey, the general and specific characteristics which are important in the assessment of its quality and indirectly its authenticity are moisture, free acidity, electrical conductivity, diastase activity, sugar, and hydroxymethylfurfural (HMF) content. Also, the labels on honey packaging could be supplemented to include information on the product′s botanical and regional origin, which is a guarantee of the quality and properties of honey and, therefore, its economic value. Unfortunately, similar to other foodstuffs of valuable nutritional and medicinal quality, honey is often subjected to either direct or indirect adulteration (21). Direct adulteration means the addition of foreign substance, *i.e*. some sugar syrup, directly to honey, while indirect adulteration of honey implies feeding honeybees with industrial sugars. In that sense, honey authentication, *i.e.* accurate determination of its composition and origin, is essential for the protection of both consumers and beekeepers.

The authenticity of honey has two aspects: authenticity with respect to honey production (addition of sugars, removal or adding of water, heating) and authenticity with respect to denomination, *i.e*. determination of botanical and geographical origin of honey (22).

The classic approach to reliable determination of unifloral honey′s botanical origin includes three complementary methods: sensory, melissopalynological, and physicochemical analysis (European Union Legislation (2001/110/EC).

Melissopalynology, *i.e*. pollen analysis by light microscopy, is the oldest method used to determine the botanical origin of honey (23). This method considers qualitative and quantitative determination of pollen and honeydew elements from honey samples (24, 25). Regardless of the inexpensive instrumentation, melissopalynology has numerous disadvantages and limitations such as the need for highly specialised personnel, great seasonal variation in pollen amount or the fact that pollen may be added fraudulently (26).

Sensory analysis of honey is considered one of the main tools for the assessment of honey's quality and botanical origin. The fact is that for many types of honey (lime honey, *Tilia* sp., for instance) physicochemical analyses do not provide enough characteristic values and the expressed hypo-pollinate features of the plant itself lower significantly the representativeness of the melissopalynological analysis. In the case of honeys with similar features (lime, sage), due to the known limitations of the melissopalynological analysis (30, 31), it is necessary to consider all parameters of quality and more attention should be paid to the organoleptic analysis (27). The results of sensory analysis may also indicate different forms of honey adulteration: the cases where honey is produced neither from nectar nor from honeydew (when sugar or sucrose solution is added or other products that have similar composition and consistency of honey, *e.g*. fructose syrup) or cases where adulterated honey is obtained by feeding bees with sugar or sugar products, or by mixing natural honey with those obtained in this way. Furthermore, it is useful in the determination of inappropriate labelling of honey relative to its botanical origin and in the detection of defects regarding the exposure of honey to temperatures above the permissible limit for the purpose of quick decrystallisation (28). Generally, the confirmation of honey′s botanical source is based on the detection and identification of the characteristic composition and typical properties in relation to the established honey standard (22, 32-34). Sometimes, the presence of a very small amount of "foreign" nectar of intense aroma may cause a serious defect of taste and odour. When identification of defects is considered, the sensory analysis of honey consents the recognition of contamination with foreign substances such as mothballs (*p*-diclorbenzene), essential oils (thymol), repellents (benzaldehyde), smell and taste of smoke, thymol, metallic taste, etc. (28, 35). Higher-quality unifloral honey is honey which, with regard to the specific features of odour, taste, appearance, and tactile properties, is as close as possible to the hypothetical honey "standard", obtained entirely from the respective plant species (27). In reality, this standard actually does not exist, except only in the

minds of trained tasters who draw the image of a perfect honey from his/her own experience (36).

The aforementioned characteristics of the classic approach to determination of the authenticity of honey indicate that it is time-consuming and requires highly skilled personnel specialised in carrying out pollen and sensory analysis (37). In order to overcome these limitations, many new methods for the easier and more reliable evaluation of authenticity of honey have been proposed in the last few decades. These methods include determination of physicochemical parameters (38), carbohydrate profiles (39), mineral content (40), amino acid composition (41), volatile organic compounds content (42, 43), polyphenolic profiles (44) or stable isotope ratios (45, 46). In order to suggest the criteria for reliable characterisation and classification of honey, several chemometric techniques such as principal component analysis (PCA), cluster analysis (CA), and linear discriminant analysis (LDA) have been used (23, 47). PCA and CA belong to the so-called unsupervised pattern recognition techniques commonly used at the introductory level of chemometric analysis in order to reduce the dimensionality of multivariate data. They provide a quick preview of the data structure, identify outliers and similar groups or clusters of the objects and, hence, simplify further data analysis (48). On the other hand, LDA falls into a group of the so-called supervised pattern recognition techniques, which aim to build mathematical models that can be used in further classification of unknown samples.

Authenticity of Serbian honey

Due to favourable relief and climatic conditions, as well as a variety of botanical species including endemics (49), Serbia has an enormous apicultural potential (50) and a very long tradition of beekeeping. In the last five years, this resulted in 520,000 – 677,000 bee colonies and an annual production of honey of 7,000-9,000 tons (51, 52). According to the annual honey production, Serbia is comparable to countries that are in the middle of the list of EU member states such as the Czech Republic, Austria, and Portugal (53).

The most common types of unifloral honeys produced in Serbia are acacia (*Robinia pseudoacacia*), lime (*Tilia cordata*), and sunflower (*Helianthus annuus*) honey, while some rare honey types such as basil (*Ocimum basilicum*), rape (*Brassica napus*), goldenrod (*Solidago virgaurea*), and buckwheat (*Fagopyrum esculentum*) honeys are produced in smaller quantities but may be found on the Serbian market every year. However, in spite of long beekeeping tradition and considerable annual production, the authenticity of Serbian honey has not been systematically studied until recently (54).

A detailed investigation of the chemical composition, and botanical and geographical origin of Serbian honey began with the characterisation of three main unifloral types of honey: acacia, sunflower, and lime honey based on some common physicochemical parameters: moisture, electrical conductivity, free acidity, specific rotation, and pH value (54). The goal of this study was to establish the possible criteria for the classification and differentiation of various botanical types of genuine Serbian honeys and, therefore, contribute to the overall investigation of European unifloral honeys (34). A total of 201 honey samples collected from seven regions of Serbia (54) were analysed using the Harmonised Methods of the International Honey Commission (55). It was found that, in general, the results of physicochemical analyses were consistent with the results reported in the previous studies of European unifloral honey samples (34, 45, 56-58). However, there were small differences, caused by different geographical conditions as well as beekeeping practices.

In order to detect the most influential differentiation factors among the studied botanical types of honey, various univariate and multivariate statistical methods were employed. One-way analysis of variance (ANOVA) and the Kruskal-Wallis test emphasised electrical conductivity and free acidity as the most significant factors, while specific rotation and moisture showed the lowest discriminating power. Such results are consistent with earlier literature reports dealing with the analysis of unifloral honey samples from other European regions (34, 57, 59). Same results were obtained by the application of multivariate chemometric methods. Thus, PCA revealed that the most influential physicochemical parameters discriminating between sunflower and lime honey were free acidity and pH while conductivity affected the separation of lime honey from the other two types. Contrary to some publications, which pointed out higher water content in sunflower honey samples in comparison with acacia and lime honey (34), it was found that moisture should not be taken as a significant criterion for differentiation between the studied botanical types of honey. In order to establish some criteria for the prediction of botanical origin of honey based only on simple physicochemical parameters, LDA was performed. This resulted in a reliable predictive model, which demonstrated that basic physicochemical parameters can be used as a rapid and reliable tool for modelling the botanical origin of honey. Finally, an attempt to differentiate 159 acacia honey samples from five regions revealed that the determined physicochemical parameters alone were not sufficient to define their geographical origin.

An assessment of honey authenticity, especially determination of its botanical origin, based on the content of amino acids, has been the subject of a number of studies (60-66). Some of them emphasised that specific amino acid profile may be used for the assessment of honey authenticity (60-62), while other reports stated that such discrimination was incomplete and could be used for an unambiguous assignment only together with other parameters such as physicochemical ones, sugar and mineral content, etc. (63-65).

The possibility to distinguish honeys according to their botanical origin was tested by the analysis of a free amino acid profile of 192 samples of seven unifloral types of Serbian honey: acacia, lime, sunflower, rape, basil, giant goldenrod, and buckwheat, from six different regions (67). For that purpose, 17 amino acids were quantified by reversed-phase high-performance liquid chromatography with fluorescence detection. Regardless of the observed similarities in amino acid profiles of the studied honey samples of given botanical origins, some important differences were identified based on both the univariate and multivariate chemometric analyses. It was found that proline, alanine, phenylalanine, threonine, and arginine were the main amino acids present in the acacia, lime, sunflower, rape, and basil honeys. In addition, in all honey samples, a high level of essential amino acids threonine, phenylalanine, histidine, and especially the conditionally essential amino acid arginine was observed. Based on chemometric analysis (Kruskal-Wallis test, PCA and LDA), phenylalanine, alanine, serine, valine, histidine, and aspartic acid were pointed out as the most important amino acids in distinguishing the investigated honey samples based on their botanical origin. Basil honey was unambiguously separated from the rest of the samples, while in the case of acacia, lime, sunflower, and rape honeys, a satisfactory efficiency of classification was achieved.

In continuation of the systematic characterisation of Serbian honey, polyphenolic profiles of 44 unifloral honeys were analysed (68). Phenolic compounds, i.e. phenolic acids and flavonoids, are constituents of honey responsible for its health-promoting effects (69) but recently these compounds were also used to successfully classify honeys according to their botanical origin (70, 71). Indeed, polyphenolic composition of honey depends on its both botanical and geographical origin, as well as on the climatic conditions of a region. Liquid chromatography coupled with various detection techniques is the method of choice for determination of phenolic substances in different samples (72). Although the current standard is liquid chromatography with a triple quadrupole ion analyser, the modern analytical trend in the analysis of complex samples such as honey is ultra-performance liquid chromatography (UPLC) coupled with high resolution and accurate mass spectrometry, as well as the use of hybrid mass analysers (73). In order to determine a characteristic polyphenolic profile of several varieties of Serbian unifloral honey, for the first time a rapid UPLC method was developed in combination with a hybrid mass spectrometer, which consists of a linear ion trap and an orbitrap mass analyser (LTQ OrbiTrap MS) (68). This technique enabled a simultaneous qualitative and quantitative MS^n analysis based on high-resolution accurate mass measurement and data dependent experiment. In order to achieve greater sensitivity to polyphenolic components electrospray ionisation technique in negative mode was used. Separations were performed on octadecyl silica stationary phase with mobile phase, which consisted of acetonitrile, water and 0.1 % formic acid. Forty-three phenolic compounds, i.e. 31 flavonoids and 12 phenolic acids and their derivatives were identified according to the corresponding spectral characteristics: mass spectra, accurate mass, characteristic fragmentation, and characteristic retention time. Relatively high amounts of propolis-derived flavonoids, chrysin, pinocembrin, and galangin were identified in all honey samples. The studied honey samples were classified according to the botanical origin using principal component analysis which resulted in two discriminant clusters: one consisted of honeys derived from perennial plants (acacia and lime) and the other from annual plants. Based on the chemometric analysis, quercetin and eriodictyol may be considered potential floral markers of sunflower honey while *cis,trans*–abscisic acid is responsible for distinguishing acacia and lime honey from sunflower honey samples.

In recent years, Fourier-transform infrared spectroscopy (FTIR) coupled with different chemometric tools has been used as an alternative method in the assessment of food authenticity (74, 75). The potential of this rapid, low cost, and reliable technique, which requires little or no sample preparation, was also studied in determination of the botanical origin of honey (76-78). The possibility to differentiate 130 Serbian unifloral honey samples (lime, acacia, and sunflower) according to their botanical origin using attenuated total reflectance infrared spectroscopy (ATR-IR) was investigated (79). For each spectrum, 64 scans were recorded in wavenumbers between 4000 and 500 cm^{-1} and at a spectral resolution of 4 cm^{-1}. The largest variations in the spectra were observed in the spectral region characteristic for the absorption that originates from monosaccharides of honey such as fructose, glucose, and disaccharides such as sucrose (34). The obtained spectra were analysed using PCA, and the calculated principal components were then used for support vector machine (SVM) training. Based on the results of PCA, significant variations among honey samples of different botanical origin were observed. Using the SVM, a classification model was built and classification errors were acquired. The SVMDA classification model yielded promising results with a 98.6 % success classification rate. Based on these results, it can be concluded that this technique offers many possibilities for further rapid qualitative analysis of honey.

The reliable evaluation of geographical origin of honey is a difficult task, which usually entails the use of several criteria and chemical markers as well as a combination of different analytical and statistical methods. In order to characterise multifloral honey using methods easily available to a large number of analytical laboratories dealing with the authenticity control of honey, a total of 164 honey samples were collected from six regions of Serbia (80). The basic physicochemical parameters (moisture, electrical conductivity, pH, free acidity, and specific rotation), 12 minerals (K, Na, Ca, Mg, Fe, Zn, Mn, Cu, Ni, Cr, Co, and Cd), and 10 characteristic mono-, di-, and trisaccharides

(glucose, fructose, trehalose, sucrose, gentiobiose, turanose, maltose, isomaltose, melesitose, and isomaltotriose) were determined in order to establish possible relationships between the corresponding chemical variables and the honey's production zone. Based on a multivariate data analysis, Mg, K, Cu, electrical conductivity, and specific rotation were selected as useful indicators in tracing regional differences between the investigated honey samples. The most influential factors, which clearly distinguished multifloral honey samples from Zlatibor region from those originating from the rest of Serbia, were the content of potassium and magnesium, as well as higher values of electrical conductivity, pH, and free acidity. The observed unique characteristics of honey from Zlatibor were considered in accordance with soil composition, climate conditions, and the presence of particular flora.

Taking into account the importance of polyphenolics in the evaluation of the origin of honey, a total of 58 polyfloral honey samples from different regions in Serbia were studied to determine their phenolic profile, total phenolic content (TPC), and the radical-scavenging activity (RSA) (81). Solid-phase extraction (SPE) combined with reversed-phase ultra-high-performance liquid chromatography-orbitrap mass spectrometry (UHPLC-LTQ OrbiTrap MS) was used to analyse the content of flavonoids and phenolic acids in honeys. The chromatographic system consisted of a C-18 stationary phase and acetonitrile + water + 1 % formic acid as a mobile phase. This way, 36 phenolic compounds were identified: 24 flavonoids, 10 phenolic acids and their derivatives, as well as 2 abscisic acids. Fourteen of the mentioned polyphenolics were quantified by comparing retention times and MS spectra with available standards. In all instances, a somewhat large amount of the propolis-derived flavonoids, such as chrysin, pinocembrin, and galangin (82) were found.

Using suitable chemometric methods, the phenolic compounds responsible for the differentiation of honey samples according to their regional origin were identified. Thus, the honeys from Vojvodina and Zlatibor region were clearly distinguished from the rest of Serbia due to the presence of dicaffeoylquinic acid, ellagic acid, caffeic acid phenethyl ester, and chlorogenic acid.

The TPC was spectrophotometrically determined with a slightly modified Folin–Ciocalteu method (83), while the RSA of the extracts of honey samples was evaluated by a modified method of Li and co-workers (84). It was found that the average content of total phenolics was in good agreement with the literature data for multifloral honeys of the surrounding regions (85-87). In addition, significant linear correlation between TPC and RSA was established indicating that flavonoids and phenolic acids may be considered phytochemicals that account for the antioxidant potential of honey (85, 87).

Regional differences between multifloral honey samples from different parts of Serbia were confirmed by geographic information systems (GIS), *i.e.* a computer-based system to input, store, manipulate, analyse, and output spatially referenced data. (88).

In continuation of the systematic investigation of Serbian honey, the differences in phytochemical profiles, more precisely in the content of phenolic compounds and sugars, depending on the maturity of lime honey, were studied (89). For that purpose, 27 ripe and one unripe honey samples, as well as one sample of lime tree nectar were collected from two areas of Serbia: 20 samples from Fruška Gora Mountain and 7 samples from Eastern Serbia. In order to check the seasonal variability, nine samples produced by the same beekeeper at the same locality were included. Melissopalynological analysis confirmed the predominance of *Tilia* nectar in all analysed samples with an average contribution of 77 %. SPE combined with UHPLC/LTQ Orbitrap MS was used to determine the content of phenolic compounds. Chromatographic separations were performed on C-18 silica stationary phase combined with a mixture of acetonitrile, water and 0.1 % formic acid as mobile phase. A total of 16 phenolics were quantified using the available standards. Similar phenolic compounds were identified and quantified in unripe and ripe lime tree honey, while the phenolic profile of the lime tree nectar showed notable differences. To be precise, a high amount of propolis-derived flavonoids chrysin, pinocembrin, and galangin were found in both ripe and unripe honey, while these substances were not detected in the lime tree nectar sample. Also, some differences in the phenolic profiles of samples from different regions were observed, but an evaluation of their significance requires detailed investigation. Due to the absence of standards, 15 glycosides were identified using the exact mass search and the study of fragmentation pathways described in the available literature. The notable difference between nectar and ripe honey samples was obtained as expected due to the hydrolytic enzymatic activity. In the case of readily hydrolysed flavonoid glucosydes, only their aglycones were found in ripe honey, while rutinosides and rhamnosides were detected in both ripe and unripe honey samples due to their inability to be hydrolysed by the bee saliva (90). In addition, the observed seasonal variability in the content of some phenolic compounds (gallic acid, cafeic acid, *cis*,*trans*-abscisic acid, apigenin, chrysin, pinocembrin, and galangin) indicated the importance of climatic conditions for the phenolic profile of honey.

Sugar profiles of the aforementioned lime honey samples were determined using high-performance anion-exchange chromatography with amperometric detection. Among the 12 determined sugars, the content of isomaltose, gentiobiose, and turanose indicated the differences in the analysed production stages of lime honey.

The authenticity of all honey samples included in the systematic investigation of Serbian honey presented in this article, with respect to their adulteration with C4 sugars, was confirmed by determination of the C-13/C-12 stable carbon isotopes ratio by means of a standard method (91).

CONCLUSION

Various criteria and different analytical methods may be used for the assessment of honey authenticity, *i.e.* determination of its botanical and geographical origin. In addition to the classic approach, which combines sensory, pollen, and physicochemical analysis, numerous new methods were proposed such as the determination of sugar profiles, amino acid composition, mineral content, polyphenolic profiles or volatile compounds. Coupled (hyphenated) techniques which combine chromatographic and spectral methods are especially useful. Finding the criteria for reliable characterisation and classification of honey requires the usage of sophisticated chemometric techniques such as various multivariate statistical methods.

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Conflicts of interest

The authors declare no conflict of interest.

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Moderne analitičke tehnike u procjeni izvornosti meda iz Srbije

Izvornost hrane u širem smislu znači udovoljavanje kemijskim i fizikalnim kriterijima koji su propisani zakonodavstvom. U pogledu izvornosti meda, dva su ključna aspekta: proizvodni proces i označavanje proizvoda u smislu zemljopisnog i botaničkog podrijetla. Pouzdana procjena izvornosti meda, koja uobičajeno uključuje korištenje nekoliko kriterija i kemijskih markera te kombinaciju analitičkih i statističkih (kemometrijskih) metoda, već dugo vremena zaokuplja pozornost kemijskih analitičara. Ovaj rad pruža uvid u različite kriterije i moderne metode za procjenu izvornosti meda koristeći se slučajem statistički značajnog broja uzoraka izvornog meda nekoliko botaničkih tipova iz različitih regija u Srbiji.

KLJUČNE RIJEČI: *analitičke metode; botaničko podrijetlo meda; kemijska karakterizacija; multiflorni med; uniflorni med; zemljopisno podrijetlo meda*