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Romanian Honey: Characterization and Classification

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Abstract

Making a significant contribution to the European honey trade, Romania has been lately engaged in an exhaustive process of ensuring product conformity. Both official bodies and research groups have taken part in the efforts to establish an efficient framework for characterizing and authenticating unifloral and polyfloral honey samples produced and commercialized. Innovative contributions of different Romanian scientists to the development of simple and/or effective investigation techniques are discussed, as well as the results gained in characterizing and classifying samples according to their botanical and/or geographical origin. Information on the honey production and commercialization in the last 25 years is also provided, as well as a sketch of the Romanian consumer profile.

Keywords: honey, trade, physico-chemical characterization, botanical, geographical classification

1. Introduction

In spite of the wealth of information regarding honey originating from different countries and continents, all of it available to the stakeholders connected to the production, commercialization, and consumption areas, Romanian honey has enjoyed much less attention. Given the increasing consumer attention to high-quality foodstuff and the intensive involvement of Romanian researchers in solving society-raised issues, an attentive analysis of the results obtained in the last decades is extremely necessary. It is our intention to put together key elements of the Romanian honey profile for those interested to develop new investigation pathways.
2. Honey production and market in Romania

The climatic and melliferous conditions are favourable for apiculture in Romania. Productions as high as 25,000 tonnes have been obtained in certain years, as the Food and Agriculture Organization Corporate Statistical database (FAOSTAT) signals [1]. The three major vegetation zones are the alpine, forest, and steppe [2]. Forests cover 29% of the country surface, with 218,500 ha of virgin forests. More than 69% are deciduous, oaks being present as Quercus species (Q. robur, Q. petrea, Q. pendiculiflora, Q. cerris, Q. frainetto), accompanied by Betula pendula, Fagus sylvatica, Larix, Carpinus, and Fraxinus. Robinia pseudoacacia occupies 120,000 ha, being found mainly in forest and plain areas; it also appears sporadically up to 400 m altitude. Tilia occupies around 54,100 ha in the forested area, the most massive culture of linden being located in Moldavia [3]. Coniferous trees in mountains areas cover almost 31.5% of the forest. The main species present in these realms are Picea abies, Pinus cembra, and Pinus sylvestris. In the main six Romanian regions the following species are also present: Amygdalus nana, Atemisia santonica, Chamaecytisus ratsisensis, Ruscus aculeantus, Paeonia peregrine, Syringa josikaea, and Tamus communis. Dobrogea region is rather different, characterized by vegetation elements common to the Danube Delta, including Carpinus orientalis, Frazinus pallisae, Populus alba, Q. pedunculiflora, Tilia tomentosa, and Vitis silvestris. Thorny bushes of Berberis vulgaris, Christi, Crataegus monogyna, and Paliurus spinus are very much encountered.

More than 60% of land in Romania is used for agriculture. One-third sustains permanent pastures, the rest is tillable. More than 50% of the arable land is planted with grains (wheat, oat, barley, and maize). Oilseeds occupy around 10%, mainly Brassica napus and Helianthus annuus. There are also other crops, such as soy, vegetables (potatoes, tomatoes, cucumber, onion, cabbage, carrot, pepper, and melons), sugar beet, rice, and vineyards. This is why the most common types are acacia, linden, raspberry, sunflower, mint, honeydew, chestnut, heather, or polyfloral honey.

Data on honey production in Europe is presently available from Food and Agriculture Organization Corporate Database, FAOSTAT, from 1961 until 2013; information on the European honey production is collected in Figure 1. In 1976, the production exceeded for the first time 100,000 tonnes, while in 2002, the 200,000 tonnes milestone has been reached. Production evolution has been constantly influenced by climatic conditions, agricultural practices, and honey-harvesting procedures. Their effects are visible in the production dynamic since 1961. According to the FAO data, the European production represented between 10 and 15% of the world production (Figure 2).

Romania is present in the international production statistics since 1961, contributing from 6.6 (in 1961) to 13.5% (1977) to the European production (Figure 3). Some of the political events are reflected by these numbers, such as the average 11% contribution in the 1977–1987 decade, when reported production raised as high as 14,000 tonnes. This period corresponded to the political decision to pay the national debts by intensive production of high value foods. The system confusion in the 1990 has induced a dramatic decrease of production to less than 10,000 tonnes, despite the tradition and relatively good climatic conditions. Afterwards, production has increased constantly to return to the previous levels and even exceed it, in 2003.
Figure 1. Honey production in Europe (source FAOSTAT).

Figure 2. Honey production in the 1961–2013 interval (source FAOSTAT).
The positive trend continued in the following years, and in 2013, honey production overcame a level never reported before, of 26,000 tonnes. Since then, there has been another fall below 20,000 tonnes, connected to the decrease in the honeybee colonies and pesticide-induced diseases. Such a trend has been reported for all other honey-producing countries.

A quick look to the main types produced since 2006 to the date (Figure 4) shows that the dominant polyfloral honey has varied from 30.5% (2012) to 87.5% (2006).

2012 has been an exceptional year, the sunflower honey representing 46.4% of the production, thus exceeding the polyfloral. These variations are tightly connected to the climatic conditions and the vegetative cycles of the plants on which honeybees fed. Exceptional years for acacia honey have been 2009, 2011, and 2013, when its share in the total production exceeded 21%. Along time, this has been one of the most appreciated assortments by the European consumers.

Since 2012, the EUROSTAT database provides data concerning the actors involved in organic honey production in the European Union (EU) (Figure 5). The newcomers in the Union, Romania and Bulgaria, are, along with Italy and Spain, significant suppliers of organic honey. Intensive use of pesticides in developed European countries has led to the premature death of hundreds of thousands beehives, thus leading to a decline of production.

Even if European Union represents the largest global producer of honey, it is not self-sufficient and approximately 40% of Europe’s consumption is covered with imports from other regions (Figure 6). Only Romania, Hungary, and Spain can manage a self-supply rate of 100% [5]. China and Argentina have been on the key suppliers list for a long time, together with Mexico and Thailand. China is particularly known as Europe’s main supplier of low-priced honey for industrial use and blends targeted at the mainstream market. The history of quality issues has worsened the position of Chinese honey in the global honey market, making the European Union more cautious about buying Chinese honey. As for Argentina, until a decade ago it
was Europe’s main honey supplier. Argentinean honey supplies have been affected lately by heavy loss of colonies and specialized forage. Furthermore, the European Union ruling in 2011 connected to detailed labelling and proofing that the pollen contained did not come from genetically modified crops increased the difficulties for Argentinean honey imports.

Starting with 2010, there has been a systematic increase of several percentages in the European Union honey exports. Main destinations are mature European markets in Germany, Italy, Poland, and United Kingdom, as well as some Eastern European countries. Hungary has contributed with 46% annual increase, Bulgaria with 29%, and Romania with a 26%.

The structure of trade in Romania has changed over time (Figure 7). A total of 298 tonnes of imported honey were reported in 1992, for the first time since the creation of FAO. A four times larger amount has been exported in the same year, the ratio undergoing continuous changes. 1996 stands out with a three orders of magnitude larger export of 6245 tonnes, compared with only 2 tonnes import. In the next decade, a significant increase in the import has been registered, to a maximum of 740 tonnes in 2002. This ratio between the yearly exported and imported amounts has never been achieved since, the export still exceeding the import. But in the last 5 years, imported amounts have increased steadily, so that in 2013, they reached 2967 tonnes, while exports were only 4.3 times higher.
Since 1990, the Romanian consumer has been exposed to an increasing penetration of supermarkets and advertising, while undergoing repeated swings in the socio-economic status [6]. Less than 15% of the population has enjoyed a real increase in income, while more than 20% has experienced severe falls. As a consequence, there are large segments of price-conscious consumers and developing clusters of high-income earners. Patterns of food consumption in East European countries signalled a fall as regards animal products consumption in the

Figure 5. Main actors in the organic honey production in European Union (source EUROSTAT).

Figure 6. Honey trade in Europe (data source FAOSTAT).
last 25 years and identified economic factors as the driving force responsible. Premium food products consumption has been neglected, so no information about honey in the area can be found before 2006. Arvanitoyannis and Krystallis [6] paid attention to the behaviour of the Romanian consumer as regards honey, a premium product with special dietary and health properties. They have investigated purchasing and consumption channels, preferences during the acquisition process, awareness regarding ‘organic food’, and sketched respondents’ profiles. A total of 220 respondents filled in a questionnaire regarding frequency, expenditure, and place of food purchase, mode of honey purchase and consumption, quality criteria, awareness and stated willingness to pay for organic honey and overall reasons for honey preferences and/or non-preferences. Answers revealed that in spite of changes in the eating habits (brought along by the changes in the retail commerce), honey is still a product purchased in bulk from individual beekeepers or in open markets. Motivation for purchasing laid in the dietary quality, medical benefits of regular consumption, suitability with the food consumption lifestyle, and ethical character of the product. Based on the consumer motivation to purchase, there is a ‘common honey consumer’, who uses honey regularly, a ‘younger consumer indifferent towards honey’, and an ‘enthusiastic honey consumer’, who values its therapeutic properties and is willing to pay the premium prices of the organic produce. The ‘common honey consumer’ is very keen on the price, while the ‘enthusiastic honey consumer’ is extremely attentive to the quality. Romanian consumers pay generally very low attention to the labels; content, aroma, colour, thickness, and taste represent the quality identifiers rather than warranties, such as brand name or country of origin sign (even when the product is sold in bulk). The scepticism of the Romanian consumers in connection with warranties and labels is probably linked to the long-time history of foodstuff forgery, starting with the 1980s.

Interviewing a focus group consisting of 2023 subjects from 18 cultural areas, living in three types of rural communities and four types of urban settlements in 2007 and 2010, Pocol and
Tesalios [7] have reported that 11% of the adult population does not consume honey, while 35% of the population consumes less than 750 g/year. An average consumption between 750 and 2000 g/year is acknowledged by 20%, and only 20% consume more than 2000 g/year. A correlation between age and consumption has been identified, stating that subjects in the 46–60 years category consume average and large amounts; this age range is negligible in the non-consumers category. Median age subjects (32–45) reported a normal consumption, while people below 30 consume reduced amounts of honey. These signal that status and economic determinants play an important part in honey consumption in Romania. Unfortunately, no linear dependency could be found between the amount of honey purchased and consumed and the economic and status variables, higher consumption being associated with medium-high status and income. As for cultural, demographic, and environmental variables, only age, cultural area, and nationality discriminate between categories. The authors conclude that honey in Romania is not part of the general dietary habits, being associated with a medium to high welfare.

3. Quality assurance

3.1. Legal basis of honey trade

The European Union has established food hygiene and safety regulations stricter than those in force in other regions of the world. Moreover, European buyers often apply even stricter requirements of their own, depending on the market. These can vary from composition specifications to colour and taste preferences and organic/fair trade certifications.

As honey is generally used as food, the European Union legislation on food applies to all honey present on the European Union market, locally processed and imported. The basis for food legislation is laid down in the EU General Food Law, Regulation (EC) 178/2002 [8], defining responsibilities and requirements for food business operators supplying food to the European Union. Directive (EC) 110/2001 [9] sets European requirements concerning honey quality standards and labelling. It has been amended by Directive (EC) 63/2014 [10], stating that pollen is not considered an ingredient anymore and labelling of honey originating in more than one member state or third country is compulsory. It also defines the right of the commission to set methods of analysis in order to verify the compliance with provisions of the current directive and the procedures of issuing and applying new decisions.

Requirements regarding honey composition and quality standards on the Romanian market are stated in this SR 784, parts 1 and 2 [11, 12]. Part 3 of the standard establishes the analysis methods for the sensory evaluation and quantification of the mandatory physical and chemical parameters (moisture, ash, acidity, reducing and easily hydrolysable sugars, total water insoluble matter, diastase and invertase, hydroxymethyl furfural content, colour index, electrical conductivity, and palynological evaluation) [13]. It also states the methods for determining adulteration with industrial glucose, starch, gelatine, glues, and aniline pigments. In addition to these requirements, all honey must comply with the general food and safety regulations mentioned above. The Romanian standard requires evaluation of routine
physico-chemical parameters and identification of handful of adulterants. The recommended methods for evaluation of hydroxymethyl furfural (HMF) content are based on its reaction with resorcinol in acidic conditions or with barbituric acid in the presence of the carcinogenic p-toluidine [13]. Commercial contracts, even within the European Union, may contain a larger number of quality requirements than the national standard, and any importer should comply. Limited compliance with specific regulations may restrict access to certain categories of buyers.

As botanical and geographical authentication has become a marked feature of the national and international honey trade, conformity evaluation laboratories and different research groups in Romania have taken steps to evaluate a larger portfolio of parameters to be used for the classification of honey samples, including geographical origin traceability [14–20].

As regards contaminants, the national Romanian regulations for beekeeping and honey do not give details, but on the European Union territory, the Regulation (EC) 470/2009 [21], in conjunction with the annexes of Regulation (EC) 2377/90 [22], is in function and establishes the maximum residue levels (MRLs) for use of authorized veterinary drugs (mainly antibiotics) applied to honeybees. The use of veterinary drugs containing pharmacological substances not listed in the annexes of the mentioned document is prohibited.

The systematic use of pesticides in the European agriculture has led to worrying declines in bee colonies, phenomenon known as colony collapse disorder (CCD). Following the negative trend and the extensive research by the European Food Safety Authority (ESFA) [23], the European Union has decided to ban the clothianidin, imidacloprid, and thiametoxam pesticides. The European proposal targets pesticides used in the treatment of cereals and plants attractive for bees and other pollinators.

In the European Union, there are strict guidelines concerning genetically modified organisms (GMO) used as food. The ruling issued by the European Court of Justice in September 2011 stipulated that honey with traces of pollen from genetically modified crops needed special authorization and labelling before it could be commercialized in Europe. Then European Parliament authorized the shift of pollen from the ‘constituent’ to the ‘ingredient’ category, in effect from July 2014 [10]. Therefore, honey containing genetically modified pollen should no longer be labelled as containing GMOs.

An important segment of the European market is the organic honey. Regulations have become stricter in time and European honey importers will increasingly require proof of organic certification of honey before entering this market. If honey is to be marketed as ‘organic’, it has to comply with the Council Regulation (EC) 834/2007 [24]. The specified requirements for organic beekeeping are

• beehives should be located in an area, with a radius of 3 km, which is free of contamination with chemicals from industrial complexes, airports, or main roads;
• hives should be built from natural materials;
• crops on which the honeybees feed should not have been chemically treated;
• artificial honeybee fodder should also be certified as organic;
• diseases should not be treated with veterinary medicines, only with approved organic substances;
• honeybees should not be stupefied while harvesting honey.

Honey laundering is an increasingly worrying issue and refers to the re-labelling of honey from one origin to allege that it comes from another region, perceived by honey buyers as offering better quality. There is a constant race to discover affordable markers and techniques for authenticating geographical origin, with authorities and researchers on one side and international traders on the other side. The 2011 dossier on the Chinese honey shipped to India and Thailand and re-labelled before entering the European Union and the USA has prompted for concerted measures over the world. European buyers have established a working group in the International Federation of Beekeepers’ Associations (Apimondia) with the aim to set up a consequent framework to prevent and fight unfair trading [25].

Generating more than €400 million per annum, European beekeeping sector is a significant economic player. Therefore, it is assisted by the European Union through subsidies, as laid down in Council Regulation 917/2004 [26, 27]. These subsidies are mostly directed to national apiculture programmes, which support research in the field of beekeeping and physical and chemical analysis of honey, technical assistance for trade, etc. Unfortunately, current production levels within the union are falling. This trend is characteristic mainly to Western European countries such as Belgium, France, Germany, Switzerland, the United Kingdom, and the Netherlands, but it was also spotted in the South in Italy, Greece, and Cyprus.

3.2. Physico-chemical characterization

Apart from the mandatory characteristics imposed by Standardization Association of Romania [28], different research groups have been engaged in the last 25 years in studying honey effects on the human body, setting up new analytical procedures, optimizing and validating those destined to routine operation, and building up an image as detailed as possible of its chemical and biochemical profile. Starting with 2005, a significant national financial support has contributed to the creation of a solid infrastructure for research and conformity compliance purposes. Some contributions are further presented, shedding light on the achievements obtained so far in exhaustively characterizing Romanian honey.

While the major sugars present in honey are readily accessible titrimetrically or spectrophotometrically, minor carbohydrates in Transylvanian acacia honey have been determined by liquid chromatography, along with individual phenolics [29]. An elaborate extraction procedure has been used prior to the identification and quantification by refractive index, UV, and mass spectrometry (MS) detection. Fructose and glucose, amounting to 42.4 and 31.9%, respectively, have been accompanied by 2.94% maltose, 2.16% sucrose, and 0.91% trehalose. Out of the 13 phenolic acids and flavonoids identified in the black locust honey, ferulic acid, abscisic acid, pinobanksine, pinocembrine, chrysin, and acacetine have been found in all studied sam-
bles, p-hydroxybenzoic acid, l-cinnamic acid, kaempferol, and apigenine have appeared in 50% of the samples, while vanillic acid, p-coumaric acid, and vanilline have been detected only in a quarter of the lot. This phenolic profile has been reported previously [30]. Abscisic acid with an average 16.2 mg/kg level (the highest concentration in the 13 phenolics detected) plays a major role in mediating plant adaptation to stress. Since ferulic acid and acacetine are found only in acacia honey samples, when comparison to the rest of honey samples produced in the area is carried out, they might be a candidate for the role of markers in botanical origin discrimination.

Marghitas et al. [18] were among the first to contribute to Romanian honey characterization in terms of antioxidant properties. Knowledge about phenols and flavonoids levels, as well as the radical scavenging activity completes the Romanian honey profile and helps understand and predict part of its dietary and health effects. Using a lot of 24 nectar and honeydew honey collected from beekeepers in 2005–2006, they determined the sugars profiles by high-performance liquid chromatography (HPLC), water, colour, and ash content according to the International Honey Commission recommendations [31]. The total phenolic content was accessible by a modification of Folin-Ciocalteu method, using gallic acids equivalents to report results, while the flavonoids were evaluated as quercitin equivalents in basic solution. All studied samples passed the Romanian quality requirements. The honeydew honey has higher ash content than the nectar honey samples evaluated. Melezitose is present only in the honeydew samples, being a good candidate as discriminant for honeydew. As for the fructose/glucose ratio, all samples with values below 1 were crystallized, while the rest were fluid at the moment of investigations. In the nectar honey category, sunflower samples contain the largest levels of phenols, as high as 45 mg gallic acid/100 g sample; this maximum is easily exceed by honeydew honey samples, whose content is 23–125 mg gallic acid/100 g sample. While the honeydew phenols content resembles that of other European studied samples [32], the Romanian nectar honey samples contain fewer phenols than the values reported by other groups [33]. A significant correlation between phenols and radical scavenging activity was found, which was better than the correlation between flavonoids and radical scavenging activity (0.94 as compared to 0.83). The honeydew honey presents the highest flavonoids content, the highest percent of inhibition towards free radicals, being followed by sunflower, lime, and acacia honey.

The special situation of honeydew honey has been further addressed by Chis et al. [34], when they compared the total phenolic compounds, flavonoids, and vitamin C levels in 10 samples from Bihor, Romania, and Podcarpackie, Poland, collected from beekeepers in 2012–2013. Two Polish samples were labelled organic. Apart from the attempt to standardize the evaluation procedure for radical scavenging activity using 2,2-di(phenyl-1-hydrazyl-hydrate) by using the percentage concentration of honey inducing a 50% inhibition of the free radical, IC50%, and the inhibition degree induced by a 1% honey solution, AA1%, the authors reported higher homogeneity of the evaluated parameters for the Romanian samples, compared to the Polish samples. Even if the entire Polish lot was labelled as honeydew honey, samples were different in appearance: ‘usual’ samples were dark brown, highly viscous, opaque, and completely liquid, while the ‘organic’ samples were light brown, opaque, and crystallized. The hypothesis of floral honey addition has been rejected based on the lower levels of phenolic compounds
in Polish colza and sunflower honey, the possible candidates for adulteration. Ascorbic acid, flavonoids, and polyphenols are present in significant amounts, Polish samples being richer in all three compounds. The good correlation between the polyphenols levels and the radical scavenging activity points out that polyphenols are the main contributors for the antioxidant properties of honey.

Information on the polycyclic aromatic hydrocarbons is mainly required when exporting Romanian honey on European and American markets. Nectar honey samples and other by-products (propolis, royal jelly, bee venom, bee wax) are prone to contamination by products resulted from the partial combustion of organic matter during different industrial processes, polycyclic aromatic hydrocarbons. Since many of these hydrocarbons have been proved to have mutagenic and/or carcinogenic effect [35], there has been an increasing concern about the levels of polycyclic aromatic hydrocarbons in foodstuff, not only in water, air, and soil. Investigations of Dobrinas et al. [19] lead to a successful procedure for extraction of polycyclic aromatic hydrocarbons from honey and propolis originating from 15 Romanian regions using hexane, followed by separation on aluminium oxide and silica gel chromatographic column and gas spectrophotography-mass spectrophotometry (GS-MS) dosage. Fourteen different aromatic hydrocarbons were determined,acenaphthene, and fluorine being the most abundant, at levels ranging from 2.0 to 55.0 ng/g. According to Environment Protection Agency, benzo[a]anthracene, benzo[k]fluoranthen, chrysene, benzo[a]pyrene, dibenzo[a,h]anthracene, and indenol[1,2,3-cd]pyrene are potential carcinogens. Chrysene, benzo[a]anthracene, and dibenzo[a,h]anthracene were below the limit of quantification in all samples. Benzo[k] fluoranthene, and benzo[a]pyrene varied in the 1–155 ng/g, while indenol[1,2,3-cd]pyrene appeared at levels below 23 ng/g, being absent in the samples from Deva rural area and Pecineaga. The highest level was obtained for samples from Bucharest urban area. The lowest levels were recorded in samples collected from Pecineaga and Dragasani rural areas. Samples originating from urban areas are characterized by much higher levels of the six carcinogenic polycyclic aromatic hydrocarbons. Whenever a forest has surrounded the beehives, levels of contamination have been much lower. The same has been found for propolis, so the authors have concluded that polycyclic aromatic hydrocarbons contamination of samples originating from the rural and mountain areas is significantly lower than for samples collected from urban areas. Contamination comes from atmospheric sources or from the soil on which plants grow. The levels of polycyclic aromatic hydrocarbons measured in honey and propolis are comparable with values found in grains, milk, and lettuce, lower than those found in olives. Luckily, the detected polycyclic aromatic hydrocarbons levels do not raise any concern for the human health.

How does organic honey perform from the quality parameters point of view had been reported by Badescu et al. [36] after measuring moisture, HMF, colour, and antibiotics residues of acacia, linden, and polyfloral honey samples collected in 2012–2015 from beekeepers members of the Romanian Beekeepers Association, in Bacau and Deva. Three samples were taken from each type of honey, for each year, amounting to 54 samples. Water content varied in the 17–19.5% range stating all samples as superior quality honeys. Only one acacia sample collected from Bacau region in 2014 out of 54 in the studied lot had 1.23 mg HMF/100 g samples. As for the antibiotics residues, they were not put in evidence, thus meeting the national
requirements for antibiotics residues in food stuff. It is thus gratifying that the organic honey originating from Bacau and Deva regions observe the quality standards for honey, as well as the European provision for organic honey.

Next to the routine physico-chemical parameters, Stihi et al. [37] investigated the presence of a series of metals by energy dispersive X-ray fluorescence (Ca, K) and atomic absorption spectrometry (Fe, Cu, Zn, and Pb) in 18 unifloral honey samples (acacia, lime tree, colza, and sunflower) from different sites of Romania. The quality requirements according to the national and European requirements have been fulfilled by most of the lot, with the exception of four samples, some adulteration suspicions and the likelihood of fermentation being signalled. Using an yttrium internal standard, the authors have found an average potassium level of 269.8 mg/kg in 2012 and a 271.9 mg/kg in 2013 and almost five times less calcium. Iron and copper levels have been as high as 6.46 and 3.1 mg/kg, respectively. Only six honey samples contained copper up to 2.2 mg/kg, while lead exceed the limit imposed for drinking water and foodstuff of 1 mg/kg. Results evaluation by two-tailed t test and principal component analysis demon-
strate that K, Ca, and Cu levels are connected to the honeybee activity and nectar plants visited by the honeybees, while Fe, Zn, and Pb appear as a result of air and soil pollution.

Volatile organic compounds are present in honey in very different amounts and their profile has been expected to vary with the botanical origin of the flowers supplying the nectar for honey production. Sample workup is crucial to the investigation success, so a variety of approaches has been used, such as solid phase microextraction [38], liquid-liquid extraction, static head space [39], or purge and trap [40]. Several Romanian acacia and linden honey samples, along with other samples originating from Slovakia, Serbia, Poland, Georgia, Germany, Ukraine, Czech Republic, Italy, France, Greece, and Moldavia have been subjected to two-dimensional GC-MS, the volatiles being first separated using a non-chiral stationary phase and further fed to a chromatographic system containing a chiral stationary phase [38]. Over 270 compounds have been detected: alkanes, alcohols, aldehydes, ketones, carboxylic acids, and their methyl and/or ethyl esters. Hotrienol, linalool, and linalool oxides have been present at the highest concentration levels, while α-terpineol, 4-terpineol, and isomers of lilac aldehydes have been reported at significantly lower amounts. All these compounds have been found in all investigated samples. Enantiomer ratios of these compounds have been determined by multidimensional GC, results demonstrating that distribution varies with the botanical origin. Although present at significant levels in all samples, (2R,5S)-cis-linalool oxide exceeds 80% with respect to its (2S,5R) enantiomer only in linden honey. Rapeseed, orange, acacia, and linden honey contain almost racemic mixtures of trans-linalool oxide. A slight predomination of (2R,5R)-trans-linalool oxide over its second enantiomer is observed in sunflower honey. As Italian chestnut honey present a predomination of the (2S,5S)-enantiomer of trans-linalool oxide, it results that the enantiomer ratio of trans-linalool oxide is a potential marker for sunflower and chestnut honey. The list of good candidates continues with (S)-4-terpineol marker for sunflower honey origin, (2S,2'S,5'S)-lilac aldehydes A, B, or C for orange and acacia honey. The authors recommend that a larger pool of chiral volatile organic compounds should be evaluated when botanical origin is under scrutiny. Since all enantiomeric ratios have been observed in samples regardless their country of origin, this information cannot be exploited for geographical authentication.
3.3. Pollen spectrum

Given the characteristics of the vegetation zones in the country, 77 pollen types from 35 families were found in the 54 unifloral and polyfloral honey samples studied by Dobre et al. [41]. The international melissopalynological nomenclature recommends four different terms to be used when reporting a pollen spectrum: dominant pollen is present as at least 45% of the grains counted, the accompanying pollen should be found between 15 and 45%, the important minor pollen varies in the 3–15% range and the pollen present at less than 1% is just minor pollen. The average number of pollen forms per sample varied in the 12–44 range, with an average of 37, spread in the four categories mentioned. Current botanical classification occurs solely on the pollen count, *R.* *pseudoacacia* being the dominant pollen for acacia honey (present as 5–58% from the total count), *Tilia* pollen for linden honey (28.3–88.3%), *Brassica* for colza honey (52–93%), *H. annuus* for sunflower (57.7–65.5%). The rest falls in the category of polyfloral and honeymedium honey. Accompanying pollens found are *Prunus, Quercus, Castanea sativa, Echium, Trifolium repens, Filipendula*, and *Vitis vinifera*.

The total pollen content was also investigated; it varied from 525 to 19,525 grains per gram of honey, thus placing the studied lot in the low and very low level categories. The differences in the pollen content is attributed to the climatic conditions, pollen production of the parent plant, distance between beehive and flower field, diameter of pollen grains, and even the procedure used for extraction of honey. A principal component analysis of the pollen spectrum demonstrated that 77.89% of the entire variability of the pollen spectrum is explained by the first four principal components. The main contribution in the new components comes from *B. napus, Tilia*, and *H. annuus* types of grains.

3.4. Rheological behaviour

The complex chemical composition has a large impact on the honey viscosity, as moisture, variable sugars ratios, acids, proteins, phenolics, minerals, and pigments contribute to yield a mixture with changing molecular structure. This issue has enjoyed special attention over the time, due to the part played in processing and storage operations. Crystallization is a serious issue, causing problems during the extraction, filtration, mixing, and packaging stages. As crystallization decreases with the temperature, it looks that heating may overcome some of the processing troubles, but at the same time induces hydroxymethyl furfural formation, a strictly regulated quality parameter [11, 12].

Studies have identified a temperature-dependent Newtonian behaviour for acacia, heather, sunflower, lime, and rape honey, as well as non-Newtonian behaviour for certain crystallized samples [42, 43]. Several anomalies in terms of yield point, shear thinning, and rheodynamic behaviour of the crystallized honey in the temperature range investigated have been detected. It has been concluded that crystallization is significantly affected by the botanical origin, temperature profile, and storage time. Modelling of the viscoelastic properties and their relation to moisture, palynological spectrum, and sugars have been addressed by several groups, using either domestic or European honey for study [44–48]. The declared objectives were correct prediction of the rheological behaviour and identification of further correlation with the botanical origin.
Using a set of 52 artisanal honey samples collected directly from Romanian beekeepers during the 2009–2010 flowering season, Dobre et al. [46] have verified the pollen spectrum, moisture, carbohydrate composition, and rheological parameters. Six specific carbohydrates (fructose, glucose, sucrose, maltose, melezitose, and trehalose) and rheological parameters (loss modulus and shear stress) were used as predictors in the viscosity function. It was confirmed that granulation is favoured by a glucose/fructose ratio (F/G) larger than 1.3, as it is the case with sunflower and rape, while honeys with higher fructose content present a very low crystallization rate, maintaining the liquid appearance for years (typical for black locust honey). F/G ratio favours rapid solid phase formation: crystallization is slow or absent for a ratio lower than 1.7, but becomes complete if it exceeds two. Some correlations between pollen content and each type of carbohydrate were noticed for at least 45% pollen. On the other hand, significant amounts of crystallized glucose lead to lower deformation stress values, as the molecular network is already destroyed when the shear is applied. Colza and honeydew honeys present non-Newtonian shear thinning behaviour, as viscosity decreases with increasing shear rate. This is not a surprise, as honeydew honey contains large amounts of proteins (of high molecular mass), and sunflower honey presents the highest content of carbohydrates, in line with the findings of other groups for colza [42] and heather [43] honey.

A deeper insight in the rheological behaviour of Romanian honey has been offered by Stoica-Guzun et al. [48]. They studied acacia, lime, coriander, peppermint, colza, sunflower, and polyfloral honey before and after heating at 50°C, looking for the compatibility degree with the Newtonian law of viscosity. Viscosity, Arrhenius constant at 20°C, and activation energies were measured for all unheated and heated samples. The qualitative analysis of the flow curves signalled the presence of a thixotropic behaviour for peppermint and colza honey, which diminished and even disappeared at higher temperatures. Using thixotropic relative areas (ratio of the thixotropic area to the area limited by the upper flow curves) at 30, 40, and 45°C, the authors attempted to classify honey samples using cluster analysis. Regardless the presence or absence of preheating, two clusters were formed, with cluster composition depended on the thermal regime. Thixotropy appears more often for unheated samples, but regresses with heating. The authors have pointed out that honey likely to crystallize (having higher glucose contents) are those prone to thixotropic behaviour.

The general model proposed by Oroian et al. [44] to describe the viscoelastic properties of honey is a fourth-order polynomial equation, applicable to all honey types (unifloral, polyfloral, or honeydew), for a 5–40°C temperature range. Validation on a set of Spanish honey samples having 32–42% fructose, 24–35% glucose, 79–83% reducing sugars, 16–19% water, and 3.4% sucrose demonstrated a Newtonian behaviour of all samples [45]. The loss modulus, G″, and viscosity show increase with moisture content, and decrease with temperature. The fourth-order polynomial equation described the combined effect of fructose, glucose, other sugars content, and moisture. A series of exponential and power models were analysed, to fit the experimental data.

A Spanish-Romanian research group [47] extended the crystallization tendency study on 136 unifloral honey samples (bramble, chestnut, eucalyptus, heather, acacia, colza, honeydew, lime, and sunflower) originating from Romania and north-west of Spain, by adding a new
descriptor to the customary pollen spectrum, sugars profile, and moisture: the ratio between the major carbohydrates. It has been found a close relation between the fructose/glucose, glucose/water, sum of the first two sugars and main pollen types in honey, namely B. napus, H. annuus, C. sativa, Rubus, and Eucalyptus. This demonstrates that the botanical source influences not only the sugar ratios, but also the crystallization process. Such descriptors bring in close proximity colza and sunflower samples, discriminating them from acacia, bramble, chestnut, eucalyptus, honeydew, and heather. The last two, containing less than 30% glucose and a high F/G ratio, are very unlikely to granulate.

4. Adulteration

Adulteration means addition of external chemical compounds to a food product containing naturally similar substances. With more than 200 major and minor components, and a constantly increasing market value, honey ranks high in the category of merchandises subjected to forgery. Honey adulteration can be carried out directly, by deliberately adding certain substances into it, or indirectly, by feeding the honeybees with the adulterating compound. Although most adulterating agents do not represent health hazards, any change in the composition or physico-chemical parameters values outside the standardized intervals may be classified as a fraud attempt and are to be sanctioned accordingly in the trading activities.

Mehryar and Esmaiili [49] have reviewed the normal values of principal physico-chemical honey parameters, drawing attention to adulteration possibilities and means of investigation. There are several possibilities to determine and report these parameters; they mainly refer to sugar content (total sugar, total reducing sugar, inverted sugar, fructose, glucose, fructose/glucose ratio), acidity (pH, free acidity, lactonic acidity, and total acidity), nitrogenous compounds (protein content, nitrogen content, proline content, diastase index, invertase index) phenolic compounds (total polyphenols, total flavonoids), HMF, minerals, and other trace elements, water content and water activity, viscosity, glass transition temperature, and colour. Authors point out that honey is adulterated directly by addition of inverted sugar or syrup (corn syrup, high fructose corn syrup, high fructose inulin syrup, and inverted syrup), intruders being difficult to detect by sugar analysis, as they have properties similar to those of natural honey. Many of the techniques involved in adulteration detection require specialized personnel and equipment, being prone to exceptional rather than routine analysis.

Plants, sources of substances used for indirect adulteration, are either C3 of C4 plants, a classification based on the carbon metabolism. The C3 plants are able to fix atmospheric carbon dioxide using the Calvin cycle, while the C4 plants use the Hatch-Slack cycle. C3 plants are characterized by a lower $^{13}\text{C}/^{12}\text{C}$ ratio than the C4 plants. Beet, rice, and wheat are C3 plants, whilst maize and sugarcane are C4 plants. Zabrodska and Vorlova [50] have discussed adulterant detection methods employed over the time, indirect adulteration of honey included, and botanical and geographical authentication issues. According to the national legislation [11] and European legislation, Council Regulation (EC) no. 797/2004 and Commission Regulation (EC) no. 917/2004 [26, 27] honey is defined as the product of the Apis mellifera honeybee species. Still there are other bee species, which also produce ‘honey’; yet according to the regulations in force, this cannot be considered true honey. Therefore, entomological origin is another issue.
that needs addressing and asks for some sort of regulations, especially in South American countries where *Melipona* and *Melipona seminigra merrillae* bees produce ‘honey’ with extremely high antioxidant and antimicrobial activity, but higher moisture, free fatty acids, and pollen content.

Using a set of 10 acacia honey samples from Valea lui Mihai, Bihor County, Marghitas et al. [51] have concentrated on clarifying their biochemical profile in relation to adulteration. The discussion basis comprises selected physico-chemical parameters (moisture, electrical conductivity, pH, pollen, total and free acidity, fructose, glucose, along with their sum and ratio, maltose, sucrose), phenolic and flavonoids data (total phenolic and flavonoids content, punctual levels of three phenolic acids and five free flavonoids) and elemental content (sodium, potassium, calcium, magnesium, copper, zinc, iron, and manganese). The natural variation of *R. pseudoacacia* pollen grains falls in the 21–36% range, in line with the national regulations. Phenolic acids rise to 12.11 mg/kg, ferulic acid representing 29% of the total amount; levels of *p*-coumaric and vanillic acid have been also determined, but appearance is random. Acacetine, pinobanksine, pinocembrine, and chrysin are present in all samples (0.38–2.28 mg/kg), quantified levels being characteristic to the Romanian acacia honey, lower than the European acacia studied by Tomas-Barberan et al. [30], but higher than the Croatian values reported by Kenjeric et al. [52]. Apart from offering a valuable instrument to confirm the compositional formula and lack of adulteration, the authors recommend the polyphenolics profile as starting point for geographic authentication.

Indirect adulteration has gained momentum in the 1970, when high fructose corn syrup became available at low costs. With an oligosaccharides profile very similar to that of natural honey, these syrups have been used as bees fed with little restriction; direct sugar analysis could not make any difference between honey produced by honeybees fed on natural honey and those produced by honeybees fed on solutions of industrial sugars. Within less than a decade, a sensitive and precise technique based on analysis of $^{13}$C/$^{12}$C stable isotopes ratio has been released [53], and proved to be effective for C3 and C4 sugars adulteration. The $^{13}$C/$^{12}$C isotopic ratio (or $\delta^{13}$C, %) varies with the photosynthetic paths, so that the C4 plants, present $\delta^{13}$C values ranging from –8 to –12‰, while for C3 plants it varies between –22 and –30‰. If honey has not been pampered with by syrup honeybee feeding, $\delta^{13}$C of its protein extract is very close to the value of honey itself. Dordai et al. [54] have used Eq. (1) in calculating the adulteration degree, drawing the attention on the fact that C4 syrups affect only the honey isotopic ratio, with little effect on its protein composition:

$$\text{Adulteration, } % = \frac{\delta^{13}\text{C}_{\text{protein}} - \delta^{13}\text{C}_{\text{honey}}}{\delta^{13}\text{C}_{\text{HFCS}}} \times 100 \quad (1)$$

They have used an elemental analyser coupled with an isotope ratio mass spectrometer to gain access to experimentally determined $\delta^{13}$C values for 12 samples of Romanian acacia, linden, sunflower, and polyfloral honeys, and their corresponding protein extracts. Some $\delta^{13}$C$_{\text{protein}}$–$\delta^{13}$C$_{\text{honey}}$ differences are positive, indicating no adulteration. Others present negative values (–0.06 to –0.98‰), thus leading to an apparent adulteration of 0.38 and 6.39%. Since –1‰ value (7% adulteration) is internationally accepted as critical threshold, only one of the 12 samples should be reported as adulterated up to 10.8% with high fructose corn syrup. The study gives access to an average $\delta^{13}$C value of –25.35‰ for Romanian honey, in line with values reported for other samples harvested in temperate climate areas of Europe. The authors
point out that δ\(^{13}\)C values vary with time, location, pollen content, but there is a levelling effect characteristic to the system itself. Honey is collected from more than one colony, over a period of several weeks. As the season starts, honeybees are fed with syrups, so there is high chance that the honey produced reflects the syrup isotopic ratio. Since hive population is renewed every 3–4 weeks, newer generations feed on the previously collected honey, so the adulterating effect of the syrup on the protein δ\(^{13}\)C value will quickly decrease.

The stable isotopic ratio methods for adulteration with C4 sugars is expensive in terms of time, consumables, personnel, and equipment, so the efforts of Puscas et al. [55] in developing a simple and reproducible high-performance thin-layer chromatographic method are welcome. It has been tested on some Romanian honey samples, being based on the F/G ratio and sucrose content evaluation. Using a suitable composition of ethyl acetate : pyridine : water : acetic acid, 6:3:1:0.5 volume ratios, high-performance thin-layer chromatographic aluminium silica gel sheets, a chromatographic twin through chamber, a dipping acetone solution of diphenylamine and aniline hydrochloride, and a visible light TLC visualization device, the authors have managed to validate the proposed procedure for the determination of the glucose, fructose, and sucrose levels. The newly validated method has given trustworthy results during the analysis of 15 Romanian acacia, linden, and polyfloral honey samples harvested by five individual producers. Almost half of the investigated samples have been declared adulterated with fructose from other sources than the natural ones. As F/G is 0.88, a polyfloral sample is declared adulterated with industrial glucose. When determined sucrose levels run above the admitted limit, there is an indication of adulteration by honeybees feeding with sucrose syrup. The acacia honey samples present a higher fructose/glucose ratio than the admitted value, effect of some producers' initiative to improve sensory properties by fructose addition (acacia honey being not too sweet).

EC regulation 470/2009 [21] states that honey should be free from antibiotics residues, serious health hazard agents. Antibiotics are generally used for the treatment of bacterial brood diseases produced by Paenibacillus larvae, known as American foulbrood (AFB). Even if they are effective only against the hives infestation with AFB, many beekeepers, the Romanians included, practice preventive antibiotics usage. Streptomycin, often used in veterinary medicine, opens up the human organism to deafness and kidney failure at higher concentrations, causing allergies, destroying intestinal flora, and inducing resistance of certain microorganisms at lower concentrations. So there is a multitude of antibiotics screening tests and confirmatory methods. High-performance liquid chromatography with post-column derivatization and fluorescence detection (HPLC-FD) is one of the most versatile and reliable methods in antibiotics residues analysis. Equally effective are the immunochemical assay kits based on antigen-antibody interactions to detect a large variety of antibiotics. The lower rate of false-negative samples, short analysis time, simple operating procedures, good selectivity, low costs are counterbalanced by the possibility to identify and quantify a single target analyte. Cara et al. [56] have used an enzyme-linked immunosorbent assay (ELISA) test kit for streptomycin to determine the antibiotic loadings in acacia, linden, and polyfloral honey samples collected from the Romanian market and get more information on the kinetic law governing the contaminant degradation on storage in the dark and different temperatures. The method has been validated (in terms of repeatability, recovery, precision, specificity, and variation coefficient), and cross-validated by high-performance liquid chromatography with post-column derivatization and fluorescence
detection. Running a F-distribution test on the experimental results dispersions obtained by the two methods demonstrates that both sets of analysis are equally reproducible, no matter the method. No residue has been detected in the samples tested. Experiments on spiked (20 and 200 μg/kg streptomycin) honey samples in the 4–70°C temperature range, for 20 weeks revealed that degradation fits a second-order multiple linear regression model for all three types of honey.

5. Statistical methods for honey classification

As mentioned before, Romania is one of the most important honey suppliers for the national and the European honey market. The quality regulation imposed for foodstuff, honey included, often requires highly specializes investigation techniques. As beekeepers are generally spread all over the country, the botanic origin is initially recorded according to the beekeepers’ declaration. Therefore, it is of great interest to find an affordable method for honey classification, based on currently measured physico-chemical properties, to confirm the declared botanic source. In this attempt, a thorough statistical study of honey properties variability is necessary. The European Union issued regulations concerning the general and specific characteristics important in assessing authenticity: moisture, sugar content (fructose, glucose, and sucrose), free acidity, diastase activity, and HMF content. These parameters are relatively simple to measure and provide a good information value.

Chemometric methods (also known as multivariate statistical technique) allow identification of the natural clustering pattern and group variables based on similarities between samples. Their application aid in reducing the complexity of large data sets, and offer better interpretation and understanding of the data sets. In the last years, several chemometric techniques, such as principal component analysis and linear discriminant analysis were used for classification of various foodstuffs [57–60]. Principal component analysis is a multivariate technique, usually at the introductory level, permitting to reduce the dimensionality of multivariate data and to provide a preview of the data structure. It belongs to the group of so-called unsupervised pattern recognition techniques, where no assumption upon possible data clustering is considered. Linear discriminant analysis falls into the group of supervised pattern recognition techniques, and classes are assumed from the beginning. Discrimination relies on finding new co-ordinates where the original data can be projected in such a way to maximize the between-group variance with respect to within-group variance. Linear discriminant analysis results may be further used at building a classification model that could later predict the class of unknowns. Artificial neural networks, designed and trained for pattern recognition, are also used to create a tool that may be used for the identification of a given unknown honey type. The efficiency of the employed statistical tools was defined in terms of their capability to classify a large set of honey samples according to their botanic origin.

5.1. Case study: experimental data

A significant data sample of four honey types (acacia, polyfloral, linden, and colza) was collected between 2014 and 2016 and the main physico-chemical characteristics were measured:
HMF, acidity, diastase index, water content, inverted sugar, and sucrose. For each honey type, 90 samples (30 samples/year) were considered in the analysis, in total 360 data sets. The unifloral and polyfloral samples were delivered, received, and transferred to the laboratory in their original packages and kept at 20°C before analysis. Information on the botanical origin of the samples was provided by the beekeepers and later validated by pollen spectrum. Aliquots were homogenized by mixing with a glass rod, filtered through cheesecloth, and left to stand until complete clarification, in order to eliminate the incorporated air, as recommended in SR 784-3:2009 [13]. Physico-chemical parameters were analysed according to the national standard [13], as presented in the literature [60]. Table 1 presents the means and ranges for all measured characteristics.

According to data recorded in Table 1, some general features can be underlined in accordance with general European Union regulations issued on the specific honey characteristics important in assessing authenticity and quality. Moisture is considered one of the basic parameters in evaluating the honey quality. According to Council Directive 2001/110/EC and Revised Codex Standard for Honey, water content may not be greater than 20%. As seen in Table 1, all honey types in the data set fulfil the quality requirements. The HMF content is indicative of honey freshness and/

<table>
<thead>
<tr>
<th>Honey type</th>
<th>Year</th>
<th>Range</th>
<th>Water,%</th>
<th>HMF mg/100 g honey</th>
<th>Diastatic index</th>
<th>Inverted sugar, %</th>
<th>Sucrose,%</th>
<th>Acidity mL 1N NaOH/100 g honey</th>
</tr>
</thead>
<tbody>
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<td>Colza</td>
<td>2014</td>
<td>Max</td>
<td>19.8</td>
<td>1.76</td>
<td>38.5</td>
<td>80</td>
<td>3.1</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min</td>
<td>17</td>
<td>0.11</td>
<td>17.9</td>
<td>75.5</td>
<td>1.15</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Average</td>
<td>18.05</td>
<td>0.61</td>
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<td>77.68</td>
<td>2.13</td>
<td>1.75</td>
</tr>
<tr>
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<td>Max</td>
<td>19.2</td>
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<td>38.5</td>
<td>80.27</td>
<td>2.88</td>
<td>2.3</td>
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<tr>
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<td></td>
<td>Min</td>
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<td>17.9</td>
<td>76</td>
<td>1.17</td>
<td>1.3</td>
</tr>
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<td>Average</td>
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<td>2.17</td>
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<td>Average</td>
<td>16.83</td>
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<td>3.68</td>
<td>1.27</td>
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<td>75.73</td>
<td>4.95</td>
<td>1.9</td>
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<td>14</td>
<td>0.09</td>
<td>10.9</td>
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<td>17.22</td>
<td>73.50</td>
<td>3.76</td>
<td>1.23</td>
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</table>
or overheating. The HMF content should not exceed 4 mg/100 g honey, but in some countries, as Germany or Romania, the maximum admitted value is lower, 1.5 mg HMF/100 g being the limit for unifloral honey samples. There are only about 5–8% individual samples in each honey type characterized by HMF values higher than 1.5 mg/100 g, thus raising possible freshness questions. The diastase activity is also indicative of freshness and is above 17 in all honey samples. Both HMF and diastase activity values determined are typical for unprocessed honey. The free acidity also varied among the four honey types investigated, but in all samples the acidity is below 4 mL NaOH solution, which is the upper limit admitted. Sugars practically consist of inverted sugar and sucrose. SR EN 784/2:2009 [12] regulates the minimum allowed inverted sugar to 70% in the flower honey. As for sucrose, the standard sets the limits to maximum 5%. All samples involved in the present study fulfill the inverted sugar and sucrose requirements (Table 1).

### Table 1. Ranges of experimental values for honey physico-chemical characteristics.

<table>
<thead>
<tr>
<th>Honey type</th>
<th>Year</th>
<th>Range</th>
<th>Water, %</th>
<th>HMF mg/100 g honey</th>
<th>Diastatic index</th>
<th>Inverted sugar, %</th>
<th>Sucrose,%</th>
<th>Acidity mL 1N NaOH/100 g honey</th>
</tr>
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<tbody>
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<td>Linden</td>
<td>2014</td>
<td>Max</td>
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<td>3.11</td>
<td>38.50</td>
<td>77.00</td>
<td>4.00</td>
<td>4.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Min</td>
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<td>0.19</td>
<td>17.90</td>
<td>72.00</td>
<td>1.44</td>
<td>1.00</td>
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<td>74.03</td>
<td>2.86</td>
<td>2.24</td>
</tr>
<tr>
<td></td>
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<td>19.00</td>
<td>2.76</td>
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<td>4.75</td>
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<td>76.94</td>
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<td>78.50</td>
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<td>72.34</td>
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<td>Average</td>
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<td>1.07</td>
<td>31.13</td>
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<td>50.00</td>
<td>79.23</td>
<td>4.27</td>
<td>3.90</td>
</tr>
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<td>Min</td>
<td>14.50</td>
<td>0.19</td>
<td>13.90</td>
<td>72.50</td>
<td>1.42</td>
<td>1.20</td>
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<td></td>
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<td>Average</td>
<td>16.64</td>
<td>1.32</td>
<td>30.24</td>
<td>75.93</td>
<td>2.76</td>
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</table>

5.2. Case study: statistical analyses

In the first stage of statistical analysis, the measured data were investigated using descriptive statistic tools and one-way analysis of variance (ANOVA) factor analysis. A first attempt was
to investigate whether the year of collection can be considered a factor that influences the honey physico-chemical properties or not. A one-way ANOVA test was performed for each honey type, results being summarized in Table 2.

As data in Table 2 show, the honey characteristic properties are not influenced by the year of collection. An exception is the influence upon the inverted sugar content in colza, linden, and polyfloral honey, and upon the HMF in the linden honey. As the time period investigated was rather short, and climatic condition were similar, the ANOVA results obtained, considering the collection year a possible influencing factor, are not unexpected.

For further statistical analysis, the data collected for each honey type in the 3 years mentioned were lumped together. Descriptive statistics tools were further used for univariate distribution analysis of each honey group. The mean, variance, skewness, and kurtosis were calculated from the data samples to evaluate the lack of symmetry and the flatness in the experimental data sets (Table 3).

As it can be noticed, the univariate distributions for all six characteristics can be considered normal for all honey types as, according to a rule of thumb generally accepted, the skewness and kurtosis are mainly in the $-1$ to $+1$ range, with few values outside this range, but still between $-2$ and 2 [61]. Only the HMF distribution for acacia and polyfloral honey is an exception to this

<table>
<thead>
<tr>
<th>Honey type</th>
<th>Sucrose</th>
<th>Inverted sugars</th>
<th>Diastatic index</th>
<th>HMF</th>
<th>Acidity</th>
<th>Water</th>
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</thead>
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<tr>
<td>Colza</td>
<td>F&lt;sub&gt;test&lt;/sub&gt; 1.05</td>
<td>3.91</td>
<td>1.23</td>
<td>2.61</td>
<td>0.90</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;crit&lt;/sub&gt; 3.10</td>
<td>3.10</td>
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<td>3.10</td>
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<tr>
<td></td>
<td>p Value 0.35</td>
<td>0.023</td>
<td>0.28</td>
<td>0.078</td>
<td>0.90</td>
<td>0.56</td>
</tr>
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<td></td>
<td>Relevance No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Acacia</td>
<td>F&lt;sub&gt;test&lt;/sub&gt; 2.98</td>
<td>1.52</td>
<td>1.39</td>
<td>0.36</td>
<td>2.19</td>
<td>2.43</td>
</tr>
<tr>
<td></td>
<td>F&lt;sub&gt;crit&lt;/sub&gt; 3.10</td>
<td>3.10</td>
<td>3.10</td>
<td>3.10</td>
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<td>3.10</td>
</tr>
<tr>
<td></td>
<td>p Value 0.055</td>
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<td>0.25</td>
<td>0.69</td>
<td>0.11</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>Relevance No</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
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<td>5.56</td>
<td>0.2</td>
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</tr>
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<td></td>
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<tr>
<td></td>
<td>p Value 0.055</td>
<td>0.007</td>
<td>0.67</td>
<td>0.005</td>
<td>0.90</td>
<td>0.28</td>
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<tr>
<td></td>
<td>Relevance No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Polyfloral</td>
<td>F&lt;sub&gt;test&lt;/sub&gt; 0.87</td>
<td>7.51</td>
<td>0.21</td>
<td>0.57</td>
<td>0.86</td>
<td>0.79</td>
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<td></td>
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<td>p Value 0.41</td>
<td>0.0008</td>
<td>0.81</td>
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<td>0.42</td>
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<td>Relevance No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

Table 2. One-way ANOVA results considering as factor the honey collection year.
The higher positive skewness of the HMF distribution is caused by some honey samples (approximately 10 out of 90 samples) with higher content (between 2 and 4.9 mg/100 g honey).

To estimate the botanical origin influence upon the main measured characteristics, the one-way ANOVA was performed in the frame of EXCEL software. The factor considered in the analysis was the honey type. The tests were carried at a significance level of 0.05. The results are presented in Table 4. Results show that honey type is a factor with statistic significance in the variation of honey physico-chemical properties. Starting from this consideration, multivariate statistical analysis is expected to give more insight concerning the possibility of honey type classification using a complex mathematical treatment of all measured variables.

Table 3. Descriptive statistic estimations for the honey types investigated.

<table>
<thead>
<tr>
<th>Statistics</th>
<th>Colza</th>
<th>Acacia</th>
<th>Linden</th>
<th>Polyfloral</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMF, mg/100 g</td>
<td>Mean</td>
<td>0.75</td>
<td>0.69</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>St. deviation</td>
<td>0.48</td>
<td>0.80</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>Skewness</td>
<td>0.96</td>
<td>2.43</td>
<td>1.66</td>
</tr>
<tr>
<td></td>
<td>Kurtosis</td>
<td>0.69</td>
<td>4.29</td>
<td>2.08</td>
</tr>
<tr>
<td>Acidity, mL 1 N NaOH/100 g</td>
<td>Mean</td>
<td>1.79</td>
<td>1.23</td>
<td>2.27</td>
</tr>
<tr>
<td></td>
<td>St. deviation</td>
<td>0.31</td>
<td>0.25</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Skewness</td>
<td>0.12</td>
<td>1.34</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Kurtosis</td>
<td>-0.89</td>
<td>1.80</td>
<td>-0.58</td>
</tr>
<tr>
<td>Diastatic index</td>
<td>Mean</td>
<td>26.82</td>
<td>17.73</td>
<td>26.09</td>
</tr>
<tr>
<td></td>
<td>St. deviation</td>
<td>5.92</td>
<td>3.78</td>
<td>5.65</td>
</tr>
<tr>
<td></td>
<td>Skewness</td>
<td>0.71</td>
<td>0.29</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Kurtosis</td>
<td>-0.07</td>
<td>-0.66</td>
<td>0.22</td>
</tr>
<tr>
<td>Inverted sugar, %</td>
<td>Mean</td>
<td>77.73</td>
<td>73.16</td>
<td>74.24</td>
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<tr>
<td></td>
<td>St. deviation</td>
<td>1.06</td>
<td>1.40</td>
<td>1.76</td>
</tr>
<tr>
<td></td>
<td>Skewness</td>
<td>0.11</td>
<td>0.53</td>
<td>-0.39</td>
</tr>
<tr>
<td></td>
<td>Kurtosis</td>
<td>-0.46</td>
<td>-0.60</td>
<td>0.09</td>
</tr>
<tr>
<td>Sucrose, %</td>
<td>Mean</td>
<td>2.04</td>
<td>3.55</td>
<td>2.72</td>
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<tr>
<td></td>
<td>St. deviation</td>
<td>0.42</td>
<td>0.97</td>
<td>0.78</td>
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<tr>
<td></td>
<td>Skewness</td>
<td>0.25</td>
<td>0.10</td>
<td>-0.08</td>
</tr>
<tr>
<td></td>
<td>Kurtosis</td>
<td>-0.09</td>
<td>-1.30</td>
<td>-0.33</td>
</tr>
<tr>
<td>Water, %</td>
<td>Mean</td>
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<td>16.62</td>
<td>17.47</td>
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<tr>
<td></td>
<td>St. deviation</td>
<td>0.69</td>
<td>1.09</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Skewness</td>
<td>0.45</td>
<td>0.57</td>
<td>0.02</td>
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<tr>
<td></td>
<td>Kurtosis</td>
<td>-0.49</td>
<td>0.87</td>
<td>-0.28</td>
</tr>
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</table>
Principal component analysis, as an unsupervised method, is generally first performed as it can lead to a data reduction and highlight the measured characteristics most responsible for data variability. As the original variables have different units, the dimensionless standardized data matrix was used in principal component analysis. All computing tasks were implemented in Matlab® [62]. Principal component analysis practically defines an orthogonal linear transformation of the original data set into a new set of coordinates, named principal components. The first PC encompasses the largest data variability, the second PC the second largest variance, and so on. According to principal component analysis, the first eigenvectors of the covariance matrix correspond to the ‘directions’ of highest variability in the data set. The first three eigenvalues are larger than 1 for the data investigated, meaning that the first three PCs explain more variability in the data set than the variables themselves. The first three principal components considered explain almost 70% of the variability (PC1 reflects 32.1%, PC2 20.7%, and PC3 15.8%) as represented by the Pareto plot (Figure 8).

The bi-plot representation (Figure 9) simultaneously shows the variables represented as vectors and the points corresponding to all samples in the data set projected in the PC1-PC2 space. The coordinates of each variable are proportional to its contribution (loading) in PC1 and PC2. The samples are displayed as points normalized in [-1, 1] interval, thus only the relative position in the graphical representation is relevant. The bi-plot allows visualization of the magnitude and sign of each variable contribution in the first two PCs. For instance, sucrose and inverted sugar have opposite signs loading, indicating that PC1 distinguishes between samples with low sucrose content and high inverted sugar content, and vice versa. As Figure 9 shows, the loadings in the first PC have high values for sucrose and inverted sugar (about 0.6), signalling that these two variables account for the most variability in the data set. HMF and water content have very small loadings in PC1, but quite high ones in PC2, revealing a smaller contribution in samples variability.

In order to visualize a possible data clustering, the projection of samples in the first two principal components space is presented of Figure 10, for the data samples in the four honey types. The ellipses cover about 95% of each honey type population. As Figure 10 shows, acacia and colza honey are clearly separated on PC1 direction, where sucrose and diastase activity present the highest loadings. These two characteristics are able to differentiate between these two botanic origins. Polyfloral honey is somehow separated from acacia and colza honey on PC2 direction, meaning that the water and HMF are responsible for

<table>
<thead>
<tr>
<th>Measured characteristic</th>
<th>Sugar</th>
<th>Inverted sugars</th>
<th>Diastatic index</th>
<th>HMF</th>
<th>Acidity</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>$F$ test value</td>
<td>58.20</td>
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<td>68.64</td>
<td>7.33</td>
<td>45.71</td>
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<tr>
<td>$F$ critical value</td>
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<td>2.63</td>
<td>2.63</td>
<td>2.63</td>
<td>2.63</td>
<td>2.63</td>
</tr>
<tr>
<td>$p$ value</td>
<td>1.2E-30</td>
<td>1.5E-57</td>
<td>4.8E-35</td>
<td>8.8E-05</td>
<td>1.4E-19</td>
<td>9.4E-22</td>
</tr>
<tr>
<td>Relevance</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 4. One-way ANOVA considering as factor the honey type.

The bi-plot representation (Figure 9) simultaneously shows the variables represented as vectors and the points corresponding to all samples in the data set projected in the PC1-PC2 space. The coordinates of each variable are proportional to its contribution (loading) in PC1 and PC2. The samples are displayed as points normalized in [-1, 1] interval, thus only the relative position in the graphical representation is relevant. The bi-plot allows visualization of the magnitude and sign of each variable contribution in the first two PCs. For instance, sucrose and inverted sugar have opposite signs loading, indicating that PC1 distinguishes between samples with low sucrose content and high inverted sugar content, and vice versa. As Figure 9 shows, the loadings in the first PC have high values for sucrose and inverted sugar (about 0.6), signalling that these two variables account for the most variability in the data set. HMF and water content have very small loadings in PC1, but quite high ones in PC2, revealing a smaller contribution in samples variability.

In order to visualize a possible data clustering, the projection of samples in the first two principal components space is presented of Figure 10, for the data samples in the four honey types. The ellipses cover about 95% of each honey type population. As Figure 10 shows, acacia and colza honey are clearly separated on PC1 direction, where sucrose and diastase activity present the highest loadings. These two characteristics are able to differentiate between these two botanic origins. Polyfloral honey is somehow separated from acacia and colza honey on PC2 direction, meaning that the water and HMF are responsible for
the differentiation. Principal component analysis could not achieve a good discrimination between the honey types: the polyfloral honey completely overlap linden, and the other honey types also partially overlap as shown in Figure 10. Figure 11 presents the principal component analysis classification capability for the case when only unifloral honey (270 samples) is considered. Figure 11 shows that the overlapping of acacia, linden, and colza samples is more or less similar to the case previously described (Figure 10).

Figure 8. Principal component contribution in the data variability.

Figure 9. Bi-plot representation in the frame of principal component analysis.
As not always the directions of highest data variability are the same with those for better
data discrimination, the classification efficiency of Fisher linear discriminant analysis was
also investigated. Linear discriminant analysis considers from the beginning the data samples
grouped in classes, and projects the data onto a lower-dimensional vector space, such that the
ratio of the between-class distance to the within-class distance is maximized, thus attempting
to achieve maximum discrimination. The optimal projection is computed by applying the
eigendecomposition on the scatter matrices. The method is recommended for large data sets
and for the case when the univariate distributions are relatively close to Gaussian repartition,
which is the case for the current experimental data set. The discrimination between groups
(honey types) is presented in Figures 12 and 13. Figure 12 corresponds to the discrimination
of the four honey types that includes the polyfloral honey, while Figure 13 reflects the linear
discriminant analysis classification capacity for unifloral honey.

When comparing the representations in Figures 10 and 12, the linear discriminant analy-
sis proves to be a better classification method for the investigated unifloral honey samples.
Analysing the samples graphical representation (Figure 12), it can be noticed that while
colza and acacia samples form distinct groups, approximately 30–40% of linden and polyfloral samples are misclassified. When only unifloral samples are subjected to classification (Figure 13), about 25% of the linden samples are represented in the acacia and colza region. Even if better results were obtained compared to principal component analysis, linear discriminant analysis does not seem accurate enough to achieve classification of unifloral honey samples based on physico-chemical properties.

The pattern recognition technique using artificial neural networks should be also tested as classification tool. A neural network with 6 input nodes (the 6 physico-chemical honey characteristics), 4 output nodes (each node corresponding to a given honey group), and 12 nodes in the hidden layer was defined in the frame of Matlab® neural network toolbox. The 360 samples were divided in 252 (70%) samples for training, 54 samples (15%) for testing, and 54 samples (15%) for validation. In this way, the results obtained are reliable, and the final fitted network would be capable to assign unknown samples to a given category. The selected training algorithm was the scaled conjugated gradient. The performance was appreciated based on mean squared error evaluation.

Figure 11. Data projection of unifloral honey samples in the PC1-PC2 space.
Figure 12. Data discrimination along the first and second linear discriminant analysis functions for the four honey type samples.

Figure 13. Data discrimination along the first and second linear discriminant analysis functions for unifloral honey samples.
The best results obtained after repeated training steps are represented with the aid of the confusion matrix in Figure 14. The number of samples correctly assigned is listed in the green boxes on the diagonal of this matrix, while the red boxes contain the number of incorrect prediction. The overall incorrect assignments represented 10.3%. For individual honey types, 96.7% of acacia honey samples, 81.2% of linden samples, 98.9% colza sets, and 82.2% polyfloral ones were correctly classified.

Figure 14. Confusion matrix for unifloral and polyfloral samples classification (1–acacia, 2–linden, 3–colza, 4–polyfloral).
For unifloral honey samples classification, a similar pattern recognition artificial neural network was built, with 6 neurons in the input layer, 3 neurons in the outer layer, and 10 neurons in the hidden layer. A total of 70% of the 270 unifloral honey samples were used for training, 15% for testing, and 15% for validation. The best results obtained led to a correct group assignment with a total error of only 3.3%. For each honey type, the errors in the sample recognition were: 4.4% for acacia, 5.6% for linden, and 0% for colza (Figure 15).
This case study, as well as those published by other Romanian researchers point out the necessity to set up a comprehensive database containing parameters of honey samples from different regions and harvesting seasons, containing not only the standardized physico-chemical parameters but also details on volatile organic compounds, phenolics, flavonoids, and stable isotopic ratios. Supervised and unsupervised classification tools would benefit from such large statistic samples, allowing a higher degree of generalization for the conclusions drawn.

6. Conclusions

The complexity of honey characterization, control, and classification has been presented using a large pool of scientific evidence, brought in by many Romanian researchers. Compared to the honey from other European countries, the Romanian honey has good market qualities due to its organic character and various botanic sources responsible for the specific flavour and consistency. The original case study presented confirms the possibility of discrimination between different honey types, based only on physico-chemical properties measurements, as demanded by the quality control.

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