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Honey Analysis

Edited by Vagner de Alencar Arnaut de Toledo

HONEY ANALYSIS

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Notice

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Meet the editor

Vagner de Alencar Arnaut de Toledo completed his master's degree (1991) and PhD degree (1997) in Animal Production (with emphasis on apiculture) and postdoctoral degree in Sciences—Entomology in 2006. He is a titular professor at the State University of Maringá. He teaches beekeeping and, since 2013, meliponiculture at the same university. He was the head of the Animal Science Department (2014–2016). He was a coordinator at the University for Animal Science undergraduation course from 2010 to 2014. He had published a book, 10 chapters in books, 76 articles in specialized scientific journals and presented 71 works personally in poster or oral form in national and international events. He had received five awards and/or honors.

Contents

Foreword

Honey is a natural product produced by bees and consumed by man since prehistory. Its density and sweet taste make it a much appreciated product by consumers, and its highly complex and nutritious chemical constitution is reported to have numerous benefits for human health.

Numerous researchers all over the world now devote their research to the study of this honeybee colony product, both for therapeutic and nutritional purposes, but also for being an important geographic and botanical marker. In addition, honey has stood out as an important product for use in monitoring environmental quality due to the foraging activity of honeybees in environments contaminated with pesticides and heavy metals.

The book *Honey Analysis* has 15 chapters divided into two sections: one section that is dedicated to the analysis of bioactive compounds, physicochemical, and microbiological analysis and another that addresses techniques for the detection of residues and heavy metals. Renowned world researchers are responsible for addressing the most relevant issues on honey analysis. *Honey Analysis* is a book and the authors' approaches to analytical techniques in honey are conveyed in a practical and objective way in association with their own research reports.

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Preface

When I was invited to edit a book about honey analysis, I thought it was a great opportunity since there are almost no books with worldwide coverage of this topic. Handouts and books can be found here and there, and there is a large number of articles. A point to be highlighted is that we have Apis mellifera honeybees with a worldwide distribution and stingless bees with a more restricted distribution to neotropical climate. Therefore, in the vast majority of countries where these bees are available, there is only a regulation for Apis mellifera and not for stingless bees. Several articles on honey analysis of stingless bees try to use as comparison the established parameters for Apis mellifera. This kind of analogy is not adequate since the parameters for stingless bees are somewhat different from those set for honeybees. For the stingless bees, it is necessary to establish new rules, but this is a subject for another book.

The topic of honey analysis is very interesting because some characteristics are very different. In the Americas we have mostly Africanized honeybees that have a natural resistance to most of the diseases that affect honeybees. The Africanized honeybees do not need acaricides to be treated from varroa and, therefore, the honey does not suffer any contamination from these acaricides. However, mainly in temperate regions, European honeybees are susceptible to varroa and therefore can only produce honey with the use of acaricides to combat this mite. With this, the honey leaves with residues of the acaricides.

However, here we have been able to compile a book with chapters by authors from nine countries (Brazil, Chile, Italy, Malta, New Zealand, Poland, Romania, Serbia, and Turkey) and at least three continents (South America, Europe, and Oceania). This book deals only with honey analysis for Apis mellifera honeybees and is divided into two parts: the first one is dedicated to physical-chemical, microbiological, and bioactive compound analyses, and another part covers the therapeutic use of honey, its use as a functional food, and its antimicrobial activities. The topics discussed here are physical-chemical analysis of honey, new methods for amino acid analysis, chemical residues, heavy metals, phenolic content and bioactive components, microbiological analysis, antimicrobial activity, and honey as functional food. Also there are notions of trade and characterization of honey in these countries, presenting the reality of the local markets of these countries and their perspectives so that we can know more about the techniques used as well as the importance of this activity for each country. This may facilitate the use of innovative techniques that may enable increased competitiveness and the world honey trade.

The chapters aim to evaluate the honey quality and the distribution of flora and apiaries, and new methods that identify specific components that give honey kind of an unique signature to determine its origin and geographical identification. Although it is a healthy food

and very concentrated in sugars, the honey contains even microorganisms. These can either cause harm to the product and the consumer if obtained without good manufacturing and handling practices, or can act as prebiotics improving the entire digestive process and hence the health of bees and consumers. Technologies of creamy honey production and its advantages are also discussed.

In my opinion, we should standardize the techniques of honey analysis at the world level, a single standard, since the quality product fits into any trade, both local (within each country) and export. Statistical tools and mathematics are beginning to be used to identify samples and adulterations, including helping to reclassify some honey quality standards.

A very interesting chapter deals with biological properties, phenolic components with beneficial effects to health, and makes a comparison of Chilean honey with others better known for their therapeutic properties. This shows that we need to study and learn more about honey because the phenolic components can be used as potential chemical markers also called bioindicators. Logically associated with this, the methods available to detect heavy metals and their uses and applications in the characterization of honey as a bioindicator of environmental pollution are also discussed in another chapter.

In countries where the European honeybee predominates, it is necessary to use antibiotics and chemotherapeutics. Here analytical methods to detect residues of these drugs, including procedures to improve the cost-benefit of these techniques, are discussed.

Therefore, I hope you will enjoy reading this book's contents on the analysis of this food that can be used as a medicine. However, there is still much to be studied and researched about honey—a food so ancient and noble.

I would also like to thank the authors for their contribution in realization of this book project

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Section 1

Bioactive Compounds, Physicochemical and Microbiological Analysis

Chapter 1

Production and Trade of Honey in Selected European Countries: Serbia, Romania and Italy

Cristina Bianca Pocol, Svetlana Ignjatijević and Daniele Cavicchioli

Additional information is available at the end of the chapter

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Abstract

The beekeeping sector is very complex, because of not onlythe diversity of bee products obtained but also the environmental services through pollination. Even if its direct impact on domestic economy and trade varies across countries, at micro-level, beekeeping creates well-being for communities, providing health products for population and decent revenues for farmers. It also supports the sustainability of rural livelihoods. In this context, the research subject is the analysis of production and trade of honey in three European Union Countries—Romania, Italy and Serbia—with a goal to consider the dynamic of supply and trade of honey and deduce potential opportunities for producers. The goal of the study is to draw implication from the results obtained, suggesting the concrete measures to improve the existing situation. Trade data are examined to get a picture of honey sector trends. An entire set of trade indicators related to honey were computed over the period 2006–2015 and are presented in the chapter: value, amount, growth rate and geographic structure of export and import in the world and in selected countries for which the level of comparative advantages of exported honey was also measured using the Balassa index.

Keywords: honey, production, international trade, competitiveness

1. Introduction

Honeybees play a crucial role for the well-being of the humanity: on the one hand, they have the amazing capacity to produce honey and other secondary products (pollen, propolis, royal jelly, venom and wax) and on the other hand, they provide pollination services for plants, bringing an important contribution to the agricultural activities and food production. The

bees represent a bio barometer for the preservation of the environment. In addition, their economic, cultural and religious values are very important [1].

The European Union is one of the most important honey producers of the world. Nevertheless, according to some official bodies, production levels seem to be declining [2]. Such decrease is attributable to the diminution of honeybee colonies, which is mostly related to the colony collapse disorder, but also to imports. The number of beehives, the quantity and quality of honey obtained are the main aspects evaluated at European level, to measure the impact of supporting policies.

Beekeeping—as agricultural activity—could be considered as multifunctional, because it performs several functions that contribute to society's welfare. At macro-level, beekeeping may play an essential economic role, creating a competitive advantage through innovation and improving the balance trade. This may be particularly true in some countries, rather than in others; however, even in those areas where the contribution of honeybee products to value added and trade of food product is limited, they represent an interesting alternative or complemental entrepreneurial activity. At micro-level, in fact, beekeeping creates well-being for communities, providing health products for population and decent revenues for farmers [1]. It also supports the sustainability of rural livelihoods, as, given the relatively low investment requirements, it is more easily engaged, compared to other rural and agricultural activities.

The key functions of beekeeping are: food security, environmental function, economical function and socio-cultural function. Beekeeping contributes to ensuring food security when people have physical and economic access to honey and other honeybee products or to other typical dishes that use honey as ingredient. The availability of honeybee products depends on local/national production and the capacity of import. The access to honeybee products depends on purchasing power of consumers and proximity to the markets. The food security has two main components: the quantity and the quality.

The quality of honey is one of the main aspects related to food security function of beekeeping. Three main components are usually taken into account in the case of honey quality: the organoleptic properties, the physical and chemical properties and the hygienic aspects (the latter is usually defined "food safety").

The organoleptic properties—aspect, consistency, colour, taste and smell are evaluated using the sensory analysis. This scientific method is used to establish the botanical origin of honey and its authenticity, but also to classify and define product standards. In addition, the method helps to identify the consumer preferences for different types of honey [3]. In EU countries, the evaluation of physical and chemical parameters of honey is made according to international legislation (Codex Alimentarius Standard), to European Union Directives and to National Legislation [4]. These parameters characterise the naturalness, maturity and unaffectedness of honey [5].

Unfortunately, the quality control of honey on the international market is sometimes vulnerable. According to Strayer et al. [6], the adulteration of honey could be economically motivated and influenced by several factors such as: the decrease of domestic production, lack of identity standards, scarce of analytical methods and trade policies. In the context of global market, there is a stringent need for finding solutions to limit the repercussions of the unfair

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practices on producers and consumers. The identification and characterisation of different types of honey via quality schemes and logos represent a protective solution that creates new opportunities for producers and consumers.

Even if compositional, sensory and safety characteristics of food are essential elements of food quality, they do not necessarily exhaust this feature, that goes beyond the observable characteristics of a product. In the last decades, the concept of quality in food (this applies also to honey) has enlarged from intrinsic attributes to extrinsic ones, focussing on some features of the production process such as its social and environmental impacts, animal welfare issues and the link of the food with a certain agricultural area. The latter aspect has received particular emphasis and attention in European countries. The quality recognition of honey (as for all food products) at European Union level can be achieved by obtaining two designations: Protected Designation of Origin and Protected Geographical Indication) [7]. According to Bertozzi [8] the use of geographical name for an agricultural product date from ancient times, "honey from Sicily" being a good example in this sense.

Protected Designation of Origin and Protected Geographical Indication tools, created within the Common Agricultural Policy, help beekeepers (and all farmers) to improve the marketing of honeybee products and to guarantee their authenticity and reputation. In the same time, they help consumer to have more trust they are buying high quality products that are connected to special places. Authenticity and traceability are the main aspects in the case of Protected Designation of Origin/Protected Geographical Indication recognition process [9]. The honey authenticity is linked to the specificity of the geographical area where it is produced: environmental factors (climate, soil and flora) and human factors (beekeeping knowledge and skills, traditional/innovative practices). There are several European countries where protected honeys with Protected Designation of Origin/Protected Geographical Indication status could be found: Greece, Spain, France, Italy, Luxembourg, Malta, Poland, Portugal and Ukraine.

Honey produced in Serbia, Romania and Italy reflects the rich diversity of melliferous plants but also the particular characteristics of regions. The quality recognition of honey is a volunteer system for beekeepers that allow valorising regional honey at European Union level. Obtaining Protected Designation of Origin and Protected Geographical Indication designation is made in compliance with European Union legislation.

In Italy, there are three types of honey with Protected Designation of Origin certification [10]: "Miele della Lunigiana", registered from 2004 [11], "Miele delle Dolomiti Bellunesi" registered from 2011 [12] and "Miele Varesino", registered, in 2014 [13]. "Miele della Lunigiana" belongs to Toscana Region (central Italy) and it is reserved for two types of honey: acacia honey and chestnut honey (one of the healthiest honeys due to its mineral content, antioxidant and antimicrobial properties). "Miele delle Dolomiti Bellunesi" is produced in the mountains of Belluno (in Veneto Region, North-East Italy) and could be found for various types: wildflower, acacia, lime, chestnut, rhododendron and dandelion. It is very appreciated not only for the floral variety, but also for other qualities such as purity, wholesomeness and lengthy shelf life. "Miele Varesino" is an acacia honey from the province of Varese (Lombardy Region, North-West Italy) with a high level of purity, due to the quality of acacia trees that grows widely in this region.

For the moment, in Serbia and Romania, there is no Protected Designation of Origin/ Protected Geographical Indication certified honey, but there is a huge potential for developing this protection in the future. In the case of Romania, the Ministry of Agriculture and Rural Development encourages the Protected Designation of Origin/Protected Geographical Indication honey certification and support this process by using the following arguments: the increase of value added of Romanian types of honey, the rise of consumer trust in the reliability of beekeepers who take care of the quality of honey, the creation of a balance between supply and demand by maintaining the quality, the facilitation of traceability and controls and the acceleration to attracting European Union funds [14]. Such strategies implemented by the Ministry of Agriculture and Rural Development could significantly contribute to the development of beekeeping sector.

In the case of Serbia, there is also a huge potential for Protected Designation of Origin/ Protected Geographical Indication certification of honey and other food products. An illustrative example is "Vlasina honey". A study about the attitudes of Vlasina honey producers towards geographical indications reveals that a small part of them know about this certification system, the results indicating the need for education and information in order to familiarise beekeepers with the procedure and the advantages of Protected Designation of Origin/ Protected Geographical Indication system [15]. "Vlasina honey" is on the list of local products supported by the European Union and the Government of Switzerland to receive technical assistance for the certification procedure. The specificity of "Vlasina honey" is given by the exceptional qualities of the region: the variety of medicinal plants and the clean environment. This type of honey is unique due to its flavour and therapeutic properties. The European recognition of "Vlasina honey" will increase the competitiveness of the beekeepers' association "Matica" and will open the opportunity to sell on international markets [16].

At present, a very small quantity of honey produced in Serbia, Romania and Italy is Protected Designation of Origin/Protected Geographical Indication protected. Hence, a question arises: what other tools could be developed to measure the quality of honey produced in these three countries? The price of different types of honey could be a real barometer for evaluating the quality? Or the high demand for export of local honey demonstrates its value?

Providing a good quantitative proxy able to describe honey quality is a hard task, as such concept and perception is heterogeneous across consumers. The widely used index to approach quality attributes of a food product is its unit value (price). It is worth remembering that price differences across products may be influenced (along with preferences for quality) by other factors, for instance, production costs and disposable income of consumers; nevertheless, price remains the most available datum that may be related to product quality, even if such correlation may be variable. In the case of honey, its quality is strictly related to product differentiation: the availability of different kind of honeys enlarges the choice set of consumers, increasing their satisfaction.

The above-mentioned considerations on honey would suggest analysing and comparing price trends for a set of different kind of honey, over time and across the three countries examined. Such a comparison would allow grasping some insights on the relative quality of each honey examined, assuming some price-quality relationship. Unfortunately, this strategy cannot be

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followed, mainly for a matter of data availability on comparability across countries and over time: price data on differentiated honey typology are rarely accessible and even when present, they are usually not gathered by official statistical bodies and are discontinuous over time. It is then clear that for any attempt to renders the concept of quality are necessary data continuous over time that are gathered and processed with homogeneous criteria over countries.

Such characteristics are fully satisfied by trade data, which are available at a high level of detail. Unfortunately, the maximum level of disaggregation for which data on traded products are released refers to "natural honey", without any further specification about the typology or characteristic of that food item. Even if the lack of information on product differentiation represents a limitation in examining quality differences among honey typologies in each country, using trade data has many advantages.

Such positive aspects are mainly due to both the opportunity to observe trade movements knowing both the value of honey traded (imported and exported) and its quantity. From this information we can derive the unit values (prices) of exchanged honey. Knowing the volume of trade, along with average import and export prices is highly valuable information as it allows analysing trade flows using a set of indexes. Such indexes, developed within the traditional trade theory of comparative advantage, tell us, among others, to what extent the honey sector in each country is competitive in its export performances, compared to the whole export of the same country. Also this trade index, along with export and import prices, may be an indirect measure of quality of honey exchanged by the selected countries.

It is quite intuitive that the ability of a product (honey) to be demanded beyond its domestic market, overcoming trade cost and cultural barriers may be seen as a combination of factors like its perceived quality that meets preferences of foreign consumers. For the same reason measures of competitiveness in trade are related on one hand to honey quality and on the other to the efficiency of beekeepers (and of their bees) to yield a product that satisfy consumers beyond the domestic market. For this reason the rest of the chapter is focussed on such topic, with the twofold objective to provide a description, even though indirect, of both the quality of the honey traded and the competitiveness of beekeepers and honey sector in the selected countries (Serbia, Romania and Italy).

The research subject is the analysis of production and trade of honey in three European Union countries: Romania, Italy and Serbia, with a goal to consider the dynamic of supply and trade of honey and point out the problem faced by producers. The goal of the study is to give a practical implication to the results obtained, by proposing concrete measures to improve the existing situation.

2. Methods

In the following sections, we analysed the level and growth rate of honey production. An entire set of trade indicators related to honey were dynamically presented in the paper: value, amount, growth rate and geographic structure of export and import in the world, European Union and selected countries. The authors also measured the level of comparative advantages

of exported honey from the selected countries by using the Balassa index. Research included a 10-year period. For this purpose, there were used data from Faostat, UN Comtrade and ITC (0409 product code), but also data provided by National Statistics Bodies.

The main body of our analysis deals with computation and comparison of the honey sector competitiveness in Serbia, Romania and Italy, to measure the comparative advantage of the honey export. The existence and extent of correlations among trade indexes is also performed. The basic concept of comparative advantage was erected in 1965 and the original Balassa model is given in Ref. [17]:

$$B = \frac{\frac{X_{ij}}{X_{it}}}{\frac{X_{nj}}{X_{nt}}}, \tag{1}$$

where $\boldsymbol{X_{ij}}$ is export of product j (honey in this case) from countries (Serbia, Romania and Italy, in this analysis); $\boldsymbol{X_{it}}$ is total export of Serbia, Romania and Italy; $\boldsymbol{X_{nj}}$ is total export of honey from world and $\boldsymbol{X_{nt}}$ is total export of the world. For values $B > 1$, the comparative advantage in honey export of the country examined is revealed. In other words, there is comparative advantage in honey export by the country when the share of honey exported on total export of the country (X_{ij}/X_{it}) is bigger than the share of honey world export on total world export (X_{nj}/X_{nt}). Ref. [18] made the correction of the index of comparative advantage and he presented it as relative trade advantage (RTA). Relative trade advantage (RTA) stands for the difference between the relative advantages of export (RXA) and the relative merits of import (RMA).

$$RTA = RXA - RMA, \tag{2}$$

$$RXA = B, \tag{3}$$

$$RMA = \frac{\frac{M_{ij}}{M_{it}}}{\frac{M_{nj}}{M_{nt}}}, \tag{4}$$

where M_{ij} is import of honey from Serbia, Romania and Italy, M_{it} is total import from Serbia, Romania and Italy, M_{nj} is total import of honey from the world and M_{nt} is total import from world. The interpretation of the relative import advantage index is symmetrical with respect to the relative advantages of export (or B) Index: the country examined is relatively more "vulnerable" to honey import (compared to its entire economy) when the share of honey imported on total import of the country (M_{ij}/M_{it}) is bigger than the share of honey world import on total world import (M_{nj}/M_{nt}). Calculating more accurate comparative advantages, Ref. [18] has created another index as the natural logarithm (ln) of the relative advantages of exports and imports (ln RXA and ln RMA). The difference obtained between the relative advantages of exports and imports is the revealed competitiveness (RC) and is expressed as:

$$RC = \ln RXA - \ln RMA. \tag{5}$$

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From the above-mentioned formula, Refs. [19, 20], has developed the following, to calculate the explicit comparative advantage:

$$\mathrm{RCA} = \ln\left[\frac{X_i}{M_i}\right] \times \left[\sum_{i=1}^{n} X_i \div \sum_{i=1}^{n} M_i\right] \times 100,$$

where X is the value of export, M is value of import, index i presents honey sector.

3. Honey exports and imports of Serbia, Romania and Italy

Results of the research show that within the analysed period the value of exports and imports increased on both the global level and in the analysed countries (**Table 1**). Comparing the change in import and export along the time span we have used the average annual growth rate (g), computed as:

$$g = \left(\sqrt[n]{\frac{f}{i}}\right) - 1,$$

where f is the final value of the series (year 2015), i is the initial value of the series (2006) and n is the time length (9 years). The interpretation of this formula is: a 5% of average growth rate means that, starting from the initial value (at 2006) i, it is necessary an annual increase of 5% to obtain the final value (at 2015) f. Average annual export growth rates in value show that Serbia had the highest average growth—37.9%. However, even with such a high value, Serbia did not achieve significant results in absolute terms, so that the average annual export amounted to USD 6.5 million with considerable oscillations per years. Romania had the highest average value of export amounting to USD 38.6 million, although over the last few years, exports increased considerably, at 9% per year, on average. The value of honey exports from Italy was growing at the rate higher than Romania but far lower than Serbia (15.1% per year) with the average value of exports being USD 31.7 million. Import data, in value, shows that Romania had the highest average annual growth rate—50.2% with average annual imports of USD 3.6 million that is however lower than the export value, with positive trade balance (export-import) of USD 35 million, on average. This makes Romania a net exporter of honey in value. Serbia imported certain quantities of honey in some years; however, they were insignificant, amounting to the average of USD 76,000. Also Serbia is a net exporter with a positive trade balance of USD 65,000, on average. Italy had the highest average value of imports amounting to USD 56.9 million with a considerable increase in imports over the last few years. Unlike Romania and Serbia, Italy is a net importer, in value, of honey with the average (2006–2015) value of import exceeding by UDS 25 million the export.

Switching from values to quantity traded (**Table 2**), the research show that over 2006–2015 period the quantity of exports and imports increased on both the global level and in the selected countries. In terms of export and import quantities, Serbia had the highest average annual growth rate—32.8%. Note that, as export quantity has grown less than export value, the unit value of exported Serbian honey grown, in nominal terms, over that period. However, the average annual quantity of honey exported from Serbia falls considerably behind Romania

Exporters	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	Average	Growth rate (%)
						Exported value						
World	830,290	894,356	1294,224	1252,091	1488,906	1701,343	1768,323	2076,365	2329,733	2355,486	1599,111.7	12.3
Romania	20,593	16,322	24,933	41,802	41,953	41,230	44,593	54,572	53,919	46,020	38,593.7	9.3
Italy	12,339	16,038	19,478	17,674	31,236	32,639	38,392	59,117	46,385	43,800	31,709.8	15.1
Serbia	537	1221	2116	3220	7537	5120	12,908	14,881	8690	9670	6590	37.9
Importers						**Imported value**					**Average**	**Growth rate (%)**
World	828,801	921,846	1251,716	1281,993	1508,615	1717,049	1760,692	2037,321	2325,266	2327,362	1596,066.1	12.2
Italy	28,305	25,098	44,864	51,967	53,363	57,967	56,116	75,188	91,183	84,534	56,858.5	12.9
Romania	159	775	2006	1545	2383	3656	5144	5546	8302	6183	3569.9	50.2
Serbia	24	45	21	59	158	74	9	240	10	120	76	19.6

Table 1. Exported and imported value in period 2006–2015 (US dollar thousand).

Exporters	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	Average	Growth rate (%)
						Exported quantity tons						
World	427,991	393,733	471,037	412,160	472,577	492,269	N/A	585,045	623,878	667,599	505,143.2	5.1
Romania	9606	6255	7087	10,654	11,017	9899	11,542	12,649	11,116	10,863	10,068.8	1.4
Italy	3669	3994	4082	3454	6960	6444	8352	11,507	8093	8882	6543.7	10.3
Serbia	159	390	638	913	1916	1108	2966	3368	1804	2045	1530.7	32.8
Importers						**Imported quantity**					**Average**	**Growth rate (%)**
World	439,322	422,411	452,659	439,810	499,914	501,863	531,285	583,653	625,577	646,299	514,279.3	4.4
Italy	13,785	10,781	13,584	15,261	14,560	15,152	15,220	18,493	21,174	23,549	16,155.9	6.1
Romania	63	315	777	516	880	1067	1824	2967	2577	2450	1343.6	50.2
Serbia	8	16	3	9	43	15	1	61	0	15	17.1	7.2

Table 2. Exported and imported quantity in period 2006–2015 (tons).

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and Italy amounting to 1530 tons. Romania had the highest average value of exports amounting to 10,000 tons. Within the analysed period, only small oscillations in exported quantities are shown, which points to the fact that production and supply on international market were balanced. The quantity of honey exports from Italy was growing at the rate of 10.3% per year with average quantity of exports amounting to 6544 tons.

Average annual growth rates of the quantity of honey imports show that Romania had the highest value—50.2% with average annual imports of 1343 tons. Nevertheless, the quantity exported from Romania is higher of 8725 tons than the imported (80% of total Romanian trade of honey). Serbia imported certain quantities of honey in some years, however, they are quite insignificant amounting to the average of 17.7 tons and confirming that Serbia is a net exporter of honey. Italy had the highest average quantity of imports amounting to 16,200 tons, far bigger than its export quantity of 9600 tons, making evident that Italy is a net importer of honey.

Table 3 emerges in the analysed period that there was an increase in the average price of honey at the rate of 7% per year on the global level. Within such period, Italy reached, on average, the highest export price of honey amounting to USD 4722 per ton, with considerable increase over the last few years. Serbia was exporting honey at the average price of USD 4023 per ton and had very low growth rate within the analysed period. Romania had the lowest average export price of honey amounting to USD 3746 with an average growth rate of 7.9% per year. At the end of the first section, we discussed on the relation between quality and price, suggesting that the former may be somehow reflected in the latter. Even if this concept is reasonable and commonly accepted, this may not apply when comparing prices across countries. In other words, the three-time series of unit value of exported honey are not comparable, that in turn means that higher export prices from a country does not necessarily imply higher quality. Even if quality is a component of the export price this may be also strongly affected by inflationary dynamics and by disposable income of the partners (importers) countries where honey is exported; also transport and other trade costs may play a role in determining export price. For these reasons, time series of exported honey unit values may be interesting if compared, for the same country, over time (and not across countries).

Italy's exports represent 1.86% of world exports for this product its ranking in world exports is 17 (**Table 4**). The average distance of importing countries is 1530 km and the export concentration is 0.22. Serbia's exports represent 0.41% of world exports for this product its ranking in world exports is 32. The average distance of importing countries is 1199 km and the export concentration is 0.17. Romania's exports represent 1.96% of world exports for this product its ranking in world exports is 15. The average distance of importing countries is 1635 km and the export concentration is 0.26. Italy's imports represent 3.65% of world imports for this product its ranking in world imports is 7. The average distance of supplying countries is 2577 km and the market concentration is 0.18. Serbia's imports represent 0.01% of world imports for this product its ranking in world imports is 110. The average distance of supplying countries is 4438 km and the market concentration is 0.34. Romania's imports represent 0.27% of world imports for this product its ranking in world imports is 37. The average distance of supplying countries is 2096 km and the market concentration is 0.2.

Exporters	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	Average	Growth rate (%)
	Exported unit value US dollar/tons											
World	1940	2271	2748	3038	3151	3456	N/A	3543	3782	3565	3054.89	7.0
Romania	2144	2609	3518	3924	3808	4165	3891	4314	4851	4236	3746.00	7.9
Italy	3363	4016	4772	5117	4488	5065	4597	5137	5731	4931	4721.70	4.3
Serbia	3382	3131	3317	3527	3934	4621	4352	4418	4817	4729	4022.80	3.8

Table 3. Exported unit value US dollar/tons.

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	Importers	Exported value 2015 (USD thousand)	Trade balance 2015 (USD thousand)	Share in countries exports (%)	Exported quantity 2015	Quantity unit	Unit value (USD/unit)	Exported growth in value between 2011 and 2015 (% p.a.)	Exported growth in quantity between 2011 and 2015 (% p.a.)	Exported growth in value between 2014 and 2015 (% p.a.)	Share of partner countries in world imports (%)	Total import growth in value of partner countries between 2011 and 2015 (% p.a.)
Exported by Italy	World	43,800	−40,734	100	8882	Tons	4931	8	6	−6	100	9
Exported by Serbia	World	9670	9550	100	2045	Tons	4729	9	8	11	100	9
Exported by Romania	World	46,045	39,880	100	10,863	Tons	4239	4	1	−15	100	9
	Exporters	**Imported value 2015 (USD thousand)**	**Trade balance 2015 (USD thousand)**	**Share in Italy's imports (%)**	**Imported quantity 2015**	**Quantity unit**	**Unit value (USD/unit)**	**Imported growth in value between 2011 and 2015 (% p.a.)**	**Imported growth in quantity between 2011 and 2015 (% p.a.)**	**Imported growth in value between 2014 and 2015 (% p.a.)**	**Share of partner countries in world exports (%)**	**Total export growth in value of partner countries between 2011 and 2015 (% p.a.)**
Imported by Italy	World	84,534	−40,734	100	23,549	Tons	3590	13	13	−7	100	10
Imported by Serbia	World	120	9550	100	15	Tons	8000	11	0	1100	100	10
Imported by Romania	World	6165	39,880	100	2450	Tons	2516	16	22	−26	100	10

Table 4. List of importing markets and list of importing markets for the product for the product 0409 natural honey.

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4. Comparative advantage of honey exports of Serbia, Romania and Italy

Studying comparative advantage in exports of honey from Serbia, we applied five indexes: relative advantages of export, relative import advantage, relative trade advantage, the revealed competitiveness and the Balassa index. **Table 5** shows the indexes for all analysed years. The research found a positive comparative advantage of all five indices. Empirical research results of comparative advantage in exports of honey from Serbia in the period 2006–2015 are shown in **Table 5**.

Results of the research of comparative advantage of honey exports from Serbia show that all the five indexes have achieved positive values. The highest level has been achieved with relative export advantage, which has caused a high positive relative trade advantage. Relative import advantage has very low, however positive value and points to negative trends and the presence of some quantities in imports.

By analysing the variance (**Table 6**), we wanted to determine whether the mean variables vary in relation to the group. In Serbia case, empirical F value is 10.141 and $p = 6.13308E{-}06$, indicating that the differences between the indexes are statistically significant.

According to research conducted [19, 21, 22], we performed a correlation analysis of the obtained indexes, to examine the extent to which the indices related to the identification of comparative advantages (**Table 7**). By using Pearson (r_p) and Spearman (r_s) test of correlation, we have proved the existence of correlation between 10 paired samples, that is, found how much the Balassa index values covariate. For Serbia, we have two pairs with strong positive correlation with p-value below .01 so we can conclude that a correlation exists and the variables covariate. Test of connection of the Balassa index using Spearman formula shows that there is a correlation in three pairs with p-value below .1 and one pair with p-value below .05, so we can conclude that a correlation exists and that the variables covariate. It is interesting to conclude that there is a correlation right between relative export advantage and relative trade advantage, as well as between the found competitiveness and the found comparative advantage, which points to the conclusion that each growth in honey exports has positive effects on the growth of comparative advantage of exports.

	N	Minimum	Maximum	Mean	Std. deviation	Variance	
RXA	10	1.20	11.93	5.2830	3.40314	11.581	RXA
RMA	10	0.00	.11	.0420	.03765	.001	RMA
RTA	10	1.18	11.93	5.2440	3.39224	11.507	RTA
RC	10	3.80	7.78	5.0950	1.30715	1.709	RC
RCA	10	1.52	4.87	2.7480	1.11179	1.236	RCA

Source: ITC and calculation of the author.

Table 5. Descriptive statistics for RXA, RMA, RTA, RC and RCA indexes of exports of honey from Serbia in the period 2006–2015.

ANOVA							Indexes	t (Dt = 9)	Sig. (2-tailed)	Mean difference
	SS	df	MS	*F*	*p*-value	*F* crit	RXA	4.909	.001	5.28300
Between groups	211.2157	4	52.80392	10.14101	6.13308E−06	2.578739	RMA	3.527	.006	0.04200
Within groups	234.3136	45	5.206969				RTA	4.889	.001	5.24400
Total	445.5293	49					RC	12.326	.000	5.09500
							RCA	7.816	.000	2.74800

Table 6. ANOVA and one sample test for RXA, RMA, RTA, RC and RCA indexes for Serbia in the period from 2006 to 2015.

		Pearson correlation					Spearman's correlation				
	Pearson correlation	RXA	RMA	RTA	RC	RCA	RXA	RMA	RTA	RC	RCA
RXA	Pearson correlation	1	.268	1.000**	.537	.589	1.000	.228	1.000**	.455	.794**
	Sig. (2-tailed)		.455	.000	.110	.073		.527		.187	.006
	N	10	10	10	10	10	10	10	10	10	10
RMA	Pearson correlation	.268	1	.258	−.612	−.347	.228	1.000	.228	−.579	−.160
	Sig. (2-tailed)	.455		.472	.060	.326	.527		.527	.080	.659
	N	10	10	10	10	10	10	10	10	10	10
RTA	Pearson correlation	1.000**	.258	1	.545	.594	1.000**	.228	1.000	.455	.794**
	Sig. (2-tailed)	.000	.472		.104	.070		.527		.187	.006
	N	10	10	10	10	10	10	10	10	10	10
RC	Pearson correlation	.537	−.612	.545	1	.883**	.455	−.579	.455	1.000	.758*
	Sig. (2-tailed)	.110	.060	.104		.001	.187	.080	.187		.011
	N	10	10	10	10	10	10	10	10	10	10
RCA	Pearson correlation	.589	−.347	.594	.883**	1	.794**	−.160	.794**	.758*	1.000
	Sig. (2-tailed)	.073	.326	.070	.001		.006	.659	.006	.011	
	N	10	10	10	10	10	10	10	10	10	10

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

Table 7. Pearson (r_p) and Spearman (r_s) test of correlation indexes for Serbia.

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	N	Minimum	Maximum	Mean	Std. deviation	Variance
RXA	10	5.26	10.12	7.4340	1.53225	2.348
RMA	10	.05	.87	.4690	.27111	.073
RTA	10	4.63	9.84	6.9660	1.65515	2.740
RC	10	1.97	5.30	3.0000	.98958	.979
RCA	10	1.50	3.08	2.0330	.47070	.222

Source: ITC and calculation of the author.

Table 8. Descriptive statistics for RXA, RMA, RTA, RC and RCA indexes of exports of honey from Romania in the period 2006–2015.

In a study of comparative advantage in exports of honey from Romania, we applied five indexes: relative advantages of export, relative import advantage, relative trade advantage, the revealed competitiveness and the Balassa index, as for Serbia. **Table 8** shows the indexes for all analysed years. The research found a positive comparative advantage of all five indices. Empirical research results of comparative advantage in exports of honey from Romania in the period 2006–2015 are shown in **Table 8**.

Results of the research of comparative advantage of honey exports from Romania show that all the five indexes reached positive values (**Table 9**). The highest level has been achieved with relative export advantage, which caused a high positive trade advantage. Relative import advantage has very low, however positive value, which points to the presence of small quantities in imports. We have concluded that Serbia and Romania have similar export structure regarding the honey exports, which points to high values in comparative advantage. Analysis of variance in the case of Romania shows the following: empirical F value is 74.51792 and $p = 2.91E{-}19$, indicating that differences between groups are statistically significant, systematic.

By using Pearson (r_p) and Spearman (r_s) test of correlation, we have proved the existence of correlation between 10 paired samples (**Table 10**). For Romania, we have six pairs with strong correlation with p-value below .01 so we can conclude that correlation exists and the variables covariate.

ANOVA							Indexes	t (Dt = 9)	Sig. (2-tailed)	Mean difference
	SS	Df	MS	F	p-value	F crit	RXA	15.342	.000	7.43400
Between groups	379.2464	4	94.8116	74.51792	2.91E−19	2.578739	RMA	5.471	.000	.46900
Within groups	57.25498	45	1.272333				RTA	13.309	.000	6.96600
Total	436.5014	49					RC	9.587	.000	3.00000
							RCA	13.658	.000	2.03300

Table 9. ANOVA and one sample test for RXA, RMA, RTA, RC and RCA indexes for Romanian the period from 2006 to 2015.

		Pearson correlation					Spearman's correlation				
		RXA	**RMA**	**RTA**	**RC**	**RCA**	**RXA**	**RMA**	**RTA**	**RC**	**RCA**
RXA	Correlation	1	−.386	.988**	.574	.803**	1.000	−.261	.964**	.467	.891**
	Sig. (2-tailed)		.271	.000	.083	.005		.467	.000	.174	.001
	N	10	10	10	10	10	10	10	10	10	10
RMA	Correlation	−.386	1	−.521	−.901**	−.557	−.261	1.000	−.430	−.952**	−.430
	Sig. (2-tailed)	.271		.122	.000	.094	.467		.214	.000	.214
	N	10	10	10	10	10	10	10	10	10	10
RTA	Correlation	.988**	−.521	1	.679*	.834**	.964**	−.430	1.000	.624	.903**
	Sig. (2-tailed)	.000	.122		.031	.003	.000	.214		.054	.000
	N	10	10	10	10	10	10	10	10	10	10
RC	Correlation	.574	−.901**	.679*	1	.792**	.467	−.952**	.624	1.000	.588
	Sig. (2-tailed)	.083	.000	.031		.006	.174	.000	.054		.074
	N	10	10	10	10	10	10	10	10	10	10
RCA	Correlation	.803**	−.557	.834**	.792**	1	.891**	−.430	.903**	.588	1.000
	Sig. (2-tailed)	.005	.094	.003	.006		.001	.214	.000	.074	
	N	10	10	10	10	10	10	10	10	10	10

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

Table 10. Pearson (r_p) and Spearman (r_s) test of correlation indexes for Romania.

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One pair showed negative correlation, which points to the fact that the increase of imports, that is, relative import advantage has negative effects upon revealed competitiveness. Test of connection of the Balassa index using Spearman formula shows that there is a correlation in four pairs with *p*-value below .1, so we can conclude that correlation exists and that the variables covariate. There is also a negative correlation with relative import advantage and revealed competitiveness.

In a study of comparative advantage in exports of honey from Italy, we applied five indexes: relative advantages of export, relative import advantage, relative trade advantage, the revealed competitiveness and the Balassa index, as for Serbia and Romania. **Table 11** shows the indexes for all analysed years. In our research, we have found positive average value for relative advantages of export and relative import advantage. Empirical research results of comparative advantage in exports of honey from Italy in the period 2006–2015 are shown in **Table 11**.

Results of the research of comparative advantage of honey exports from Italy show that positive values have been achieved in two indexes, that is, relative export and import advantage have positive average values (**Table 12**). As the relative import value grows, the level of relative trade openness, as well as the revealed competitiveness and the Balassa index become negative. Analysis of variance in the case of Italy shows the following: empirical *F* value is 139.512 and *p* = 9.51E−25, indicating that differences between groups are statistically significant, systematic.

	N	Minimum	Maximum	Mean	Std. deviation	Variance
RXA	10	.43	1.04	.6360	.19540	.038
RMA	10	.75	1.57	1.1880	.25354	.064
RTA	10	−.86	−.26	−.5520	.20460	.042
RC	10	−1.06	−.34	−.6450	.24451	.060
RCA	10	−1.06	−.26	−.6260	.24139	.058

Source: ITC and calculation of the author.

Table 11. Descriptive statistics for RXA, RMA, RTA, RC and RCA indexes of exports of honey from Italy in the period 2006–2015.

	ANOVA						Indexes	*t* (Dt = 9)	Sig. (2-tailed)	Mean difference
	SS	Df	MS	*F*	*p*-value	*F* crit	RXA	10.293	.000	.63600
Between groups	29.28445	4	7.321112	139.5118	9.51E−25	2.578739	RMA	14.817	.000	1.18800
Within groups	2.36145	45	0.052477				RTA	−8.532	.000	−.55200
Total	31.6459	49					RC	−8.342	.000	−.64500
							RCA	−8.201	.000	−.62600

Table 12. ANOVA and one sample test for RXA, RMA, RTA, RC and RCA indexes for Italy in the period from 2006 to 2015.

		Pearson correlation					Spearman's correlation				
		RXA	**RMA**	**RTA**	**RC**	**RCA**	**RXA**	**RMA**	**RTA**	**RC**	**RCA**
RXA	Correlation	1	.602	.197	.669*	.741*	1.000	.503	.311	.775**	.801**
	Sig. (2-tailed)		.066	.585	.034	.014		.138	.382	.008	.005
	N	10	10	10	10	10	10	10	10	10	10
RMA	Correlation	.602	1	−.664*	−.160	−.062	.503	1.000	−.590	−.037	.024
	Sig. (2-tailed)	.066		.036	.658	.866	.138		.072	.920	.947
	N	10	10	10	10	10	10	10	10	10	10
RTA	Correlation	.197*	−.664*	1	.826**	.772**	.311*	−.590	1.000	.761*	.750*
	Sig. (2-tailed)	.585	.036		.003	.009	.382	.072		.011	.012
	N	10	10	10	10	10	10	10	10	10	10
RC	Correlation	.669*	−.160*	.826**	1	.986**	.775**	−.037*	.761*	1.000	.976**
	Sig. (2-tailed)	.034	.658	.003		.000	.008	.920	.011		.000
	N	10	10	10	10	10	10	10	10	10	10
RCA	Correlation	.741*	−.062	.772**	.986**	1	.801**	.024	.750*	.976**	1.000
	Sig. (2-tailed)	.014	.866	.009	.000		.005	.947	.012	.000	
	N	10	10	10	10	10	10	10	10	10	10

*Correlation is significant at the 0.05 level (2-tailed).
**Correlation is significant at the 0.01 level (2-tailed).

Table 13. Pearson (r_p) and Spearman (r_s) test of correlation indexes for Italy.

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By using Pearson (r_p) and Spearman (r_s) test of correlation (**Table 13**), we have shown that for Italy we have six pairs with strong correlation, at the level of $p < .01$ and .05 so we can conclude that the correlation exists and variables covariate. One pair showed negative correlation, which points to the fact that the increase in imports, that is, relative import advantage has negative effects upon revealed competitiveness. Test of connection of the Balassa index using Spearman formula shows that there is a correlation in five pairs with $p < .01$ and .05, so we can conclude that correlation exists and that the variables covariate. There is also a negative correlation with relative import advantage and revealed competitiveness (RC).

In our further research, we wanted to determine the value of importance of the difference between the comparative advantage indexes (RCA) for all three countries (**Table 14**). The variance analysis shows the following: the empirical *F* value is 62.554 and $p = 7.32E{-}11$, which points to the fact that differences between the groups are statistically significant.

Source of variation	**SS**	**df**	**MS**	***F***	***p*-value**	***F* crit**		***t* (Dt = 9)**	**Sig. (2-tailed)**	**Mean difference**
Between groups	63.21794	2	31.60897	62.55435	7.32E−11	3.354131	RCA SRB	7.816	.000	2.74800
Within groups	13.64321	27	0.505304				RCA RO	13.658	.000	2.03300
Total	76.86115	29					RCA IT	−8.201	.000	−.62600

Table 14. ANOVA test RCA index for Serbia, Romania and Italy.

Results of further analysis show that there is statistically significant difference between the levels of comparative advantage of exports between Serbia and Italy and Romania and Italy (**Table 15**). If we consider that Serbia and Romania are producing surplus in international honey trade, while for Italy, imports are more important, then the empirical results prove the absence of the comparative advantage in honey exports in Italy.

Paired samples test		**Paired differences**					**t**	**df**	**Sig. (2-tailed)**
		Mean	**Std. deviation**	**Std. error mean**	**95% confidence interval of the difference**				
					Lower	**Upper**			
Pair 1	RCA_SRB– RCA_RO	.71500	1.40725	.44501	−.29168	1.72168	1.607	9	.143
Pair 2	RCA_SRB– RCA_IT	3.37400	1.09891	.34751	2.58789	4.16011	9.709	9	.000
Pair 3	RCA_RO– RCA_IT	2.65900	.57922	.18317	2.24465	3.07335	14.517	9	.000

Table 15. Paired samples test RCA index for Serbia, Romania and Italy.

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5. Discussion and concluding remarks

Research results point to a mild increase in world production. The production increase has positive consequences in the expansion of honey exports in some European Union countries. Honey trade and competitive patterns presented in previous tables (Sections 3 and 4) have shown clearly different trends and exchange structures in the three selected countries examined (Serbia, Romania and Italy). While Serbia and Romania are net exporter, with a positive trade balance, Italy is a net importer, with negative balance given the deficit of export compared to import. Romania and Serbia differ for the trade volume (import + export) that is about seven times bigger in Romania (on average 2006–2015). Romania has a growing market and a lot of opportunities for export. Nevertheless, even if Serbia has a smaller market, the low levels of import may suggest that domestic beekeeping industry is able to cover both internal and foreign demand (even if some other causes, like trade barriers, may have a role). Such surprising low impact of import is confirmed by previous analysis on honey consumers in Serbian regions. Ref. [20], in fact, suggest: "While researching consumer attitudes, we have come to the conclusion that the majority of consumers, as many as 83%, are willing to try Fruska Gora's lime honey that is of above average quality and is certified, regardless of the fact that lime honey does not belong to the type of honey which consumers buy. The reason for this lies in the fact that consumers prefer a high quality of honey because it is linked to better taste and better healing properties of honey, which is in line with the motivations of consumers. For such a quality and certified honey, consumers are willing to pay even a 30% higher price than the average market price of lime honey."

Apparently the opposite applies to Italy whose internal demand for honey is covered to a relevant extent by imports. This may be seen as a source of potential unexplored demand to be covered by Italian beekeepers. Both, Romania and Serbia have a high coverage of imports by exports and a positive comparative advantage of export. Such evidences are also confirmed by previous studies on competitiveness of Countries in the Danube regions. For example the study [23] concluded that "In the following commodity groups in Serbia an increase of positive comparative advantage is present: milk and products, except butter and cheese; butter and other fats from milk; dairy spreads, cheese and curd, products of cereals, flour, starch; vegetables, roots and tubers, processed; fruit prepared and products; sugar, molasses and honey; chocolate and other food preparations with cocoa". In another analysis [21] have been using D'Agostino and Pearson omnibus normality test showing that the Balassa index value distribution in Romania does not deviate significantly from normality ($K2 = 2.46$ and $p = .29$).

Romania is a net exporter of honey, well known at international level. The introduction of quality standards and the certification of honey will increase the prices of commercialised honey, mostly for the external market. Beekeepers should maintain the quality of honey by preserving the environment and the traditional practices. The production of organic honey is another sector that brings a comparative advantage for Romania and creates the possibility to develop a niche market. Anyway, Romania could represent a model of good practices for Serbia to improve the competitiveness of the beekeeping sector through innovation and associative forms [24–26].

Research has also shown that the majority of European Union countries imported honey. Italy is a net importer and has a large trade deficit in terms of value and volume, even if such gap is slightly declining. Italian beekeeping industry should take actions to recover market shares of domestic demand, developing more effective promotional activities towards consumers. The conclusion of the study reveals the fact that innovation through the whole value chain is one of the key factors for increasing competitiveness of honey production and trade. Interestingly, the results of the present analysis are also confirmed and mirrored by previous studies that point out the importance of honey quality improvement as an essential way to pursue both sector innovation and product promotion.

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Chapter 2

Romanian Honey: Characterization and Classification

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Additional information is available at the end of the chapter

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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. petraea, Q. pendiculiflora, Q. cerries, 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: *Amyldalus nana, Atemisia santonica, Chamaecytisus ratisbonensis, 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 spina* 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.

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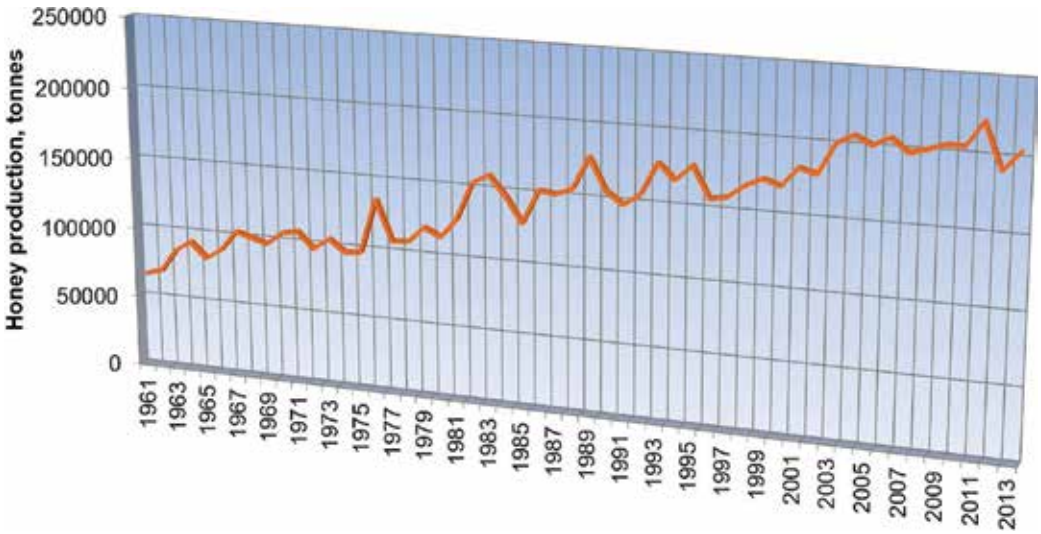

Figure 1. Honey production in Europe (source FAOSTAT).

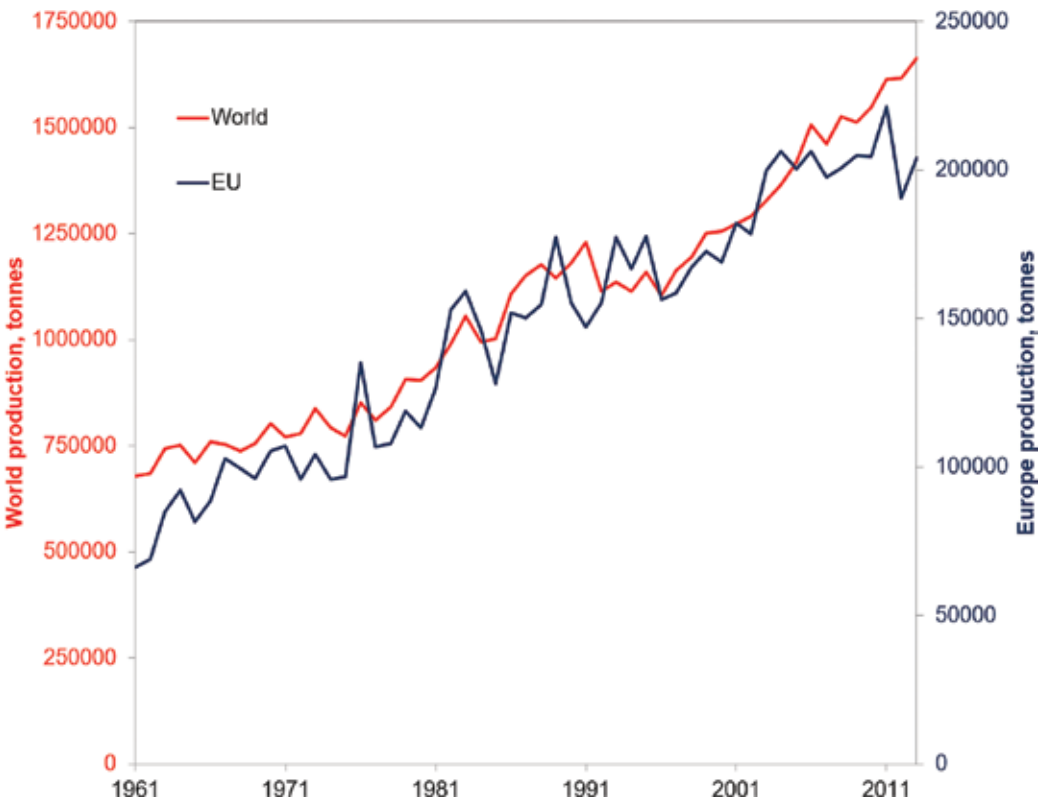

Figure 2. Honey production in the 1961–2013 interval (source FAOSTAT).

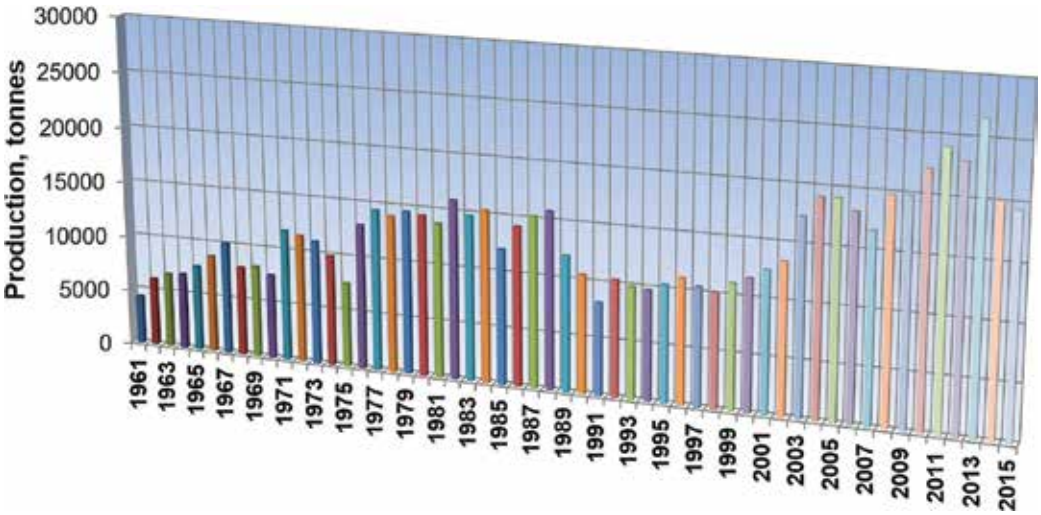

Figure 3. Honey production in Romania (data source FAOSTAT + MADR [4]).

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

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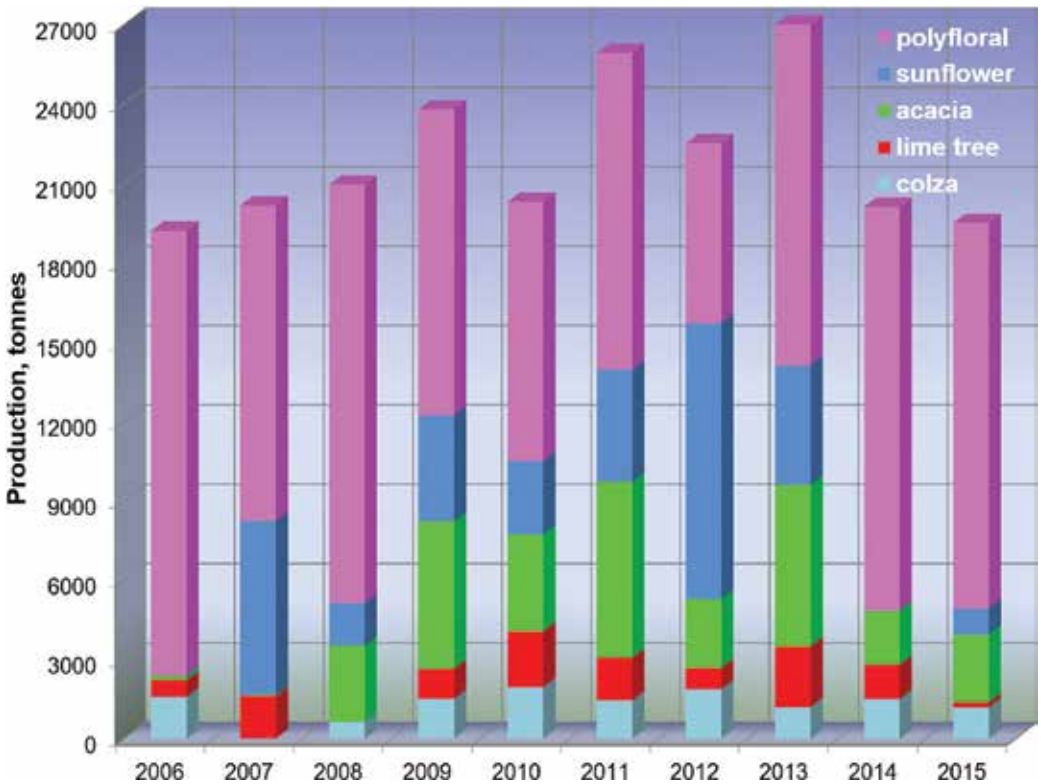

Figure 4. Romanian honey production by type (source MADR).

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.

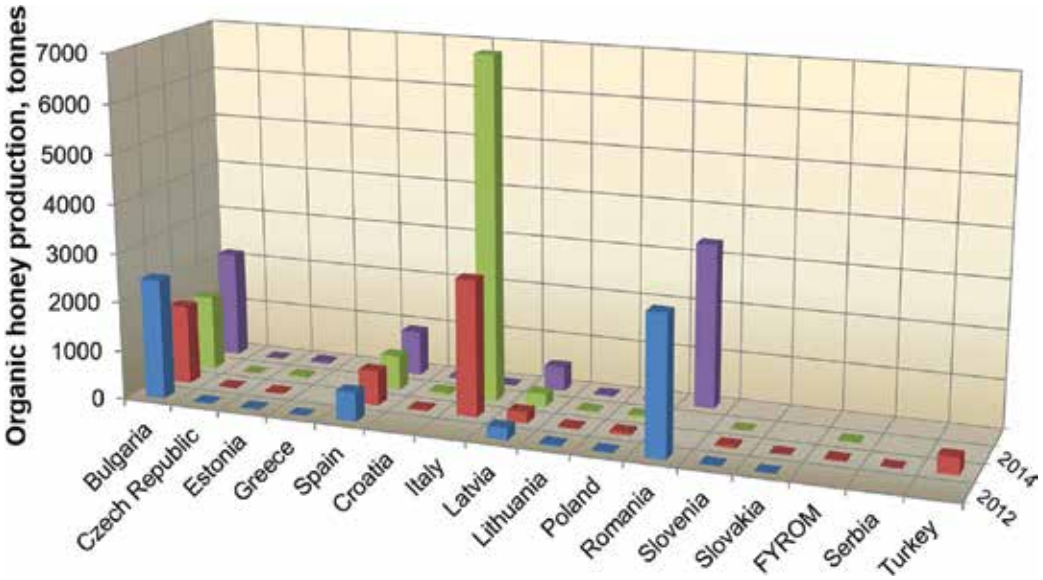

Figure 5. Main actors in the organic honey production in European Union (source EUROSTAT).

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

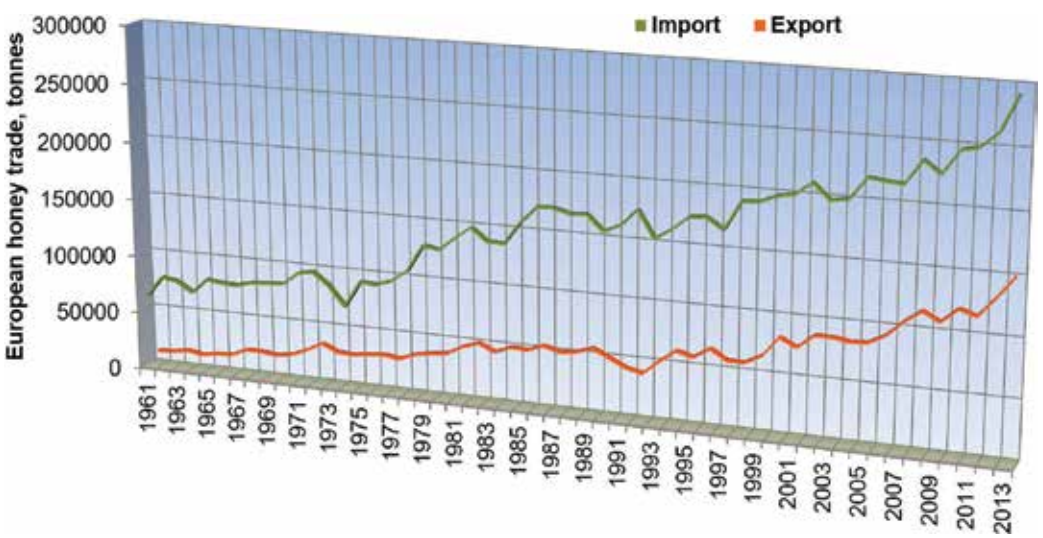

Figure 6. Honey trade in Europe (data source FAOSTAT).

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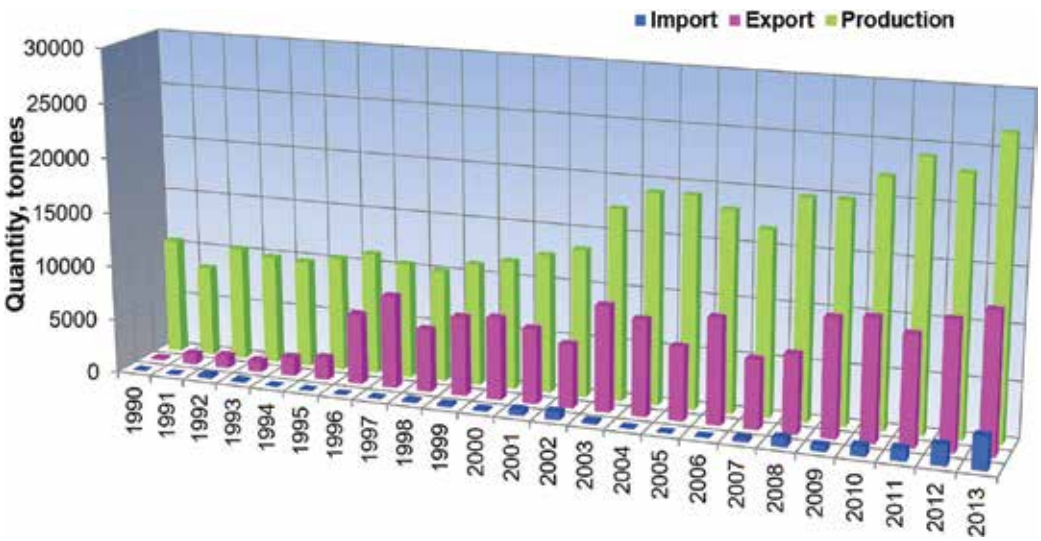

Figure 7. Romanian honey sector between 1993 and 2013 (data source FAOSTAT).

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

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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-

ples, *p*-hydroxybenzoic acid, *t*-cinnamic acid, kaempherol, 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 spectrography-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[α]anthracene, benzo[*k*]fluoranthene, chrysene, benzo[α]pyrene, dibenzo[α,*h*]anthracene, and indenol[1, 2, 3,-*cd*]pyrene are potential carcinogens. Chrysene, benzo[α]anthracene, and dibenzo[α,*h*]anthracene were below the limit of quantification in all samples. Benzo[*k*] fluoranthene, and benzo[α]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

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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-tailored *t* test and principal component analysis demonstrate 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 honeydew 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 botanic 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.

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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 presences 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 $^{13}C/^{12}C$ 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

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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, *p*H, 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:

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

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 $\delta^{13}C_{protein}$–$\delta^{13}C_{honey}$ 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 $\delta^{13}C$ 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 $\delta^{13}C$ 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

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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/

Honey type	Year	Range	Water,%	HMF mg/100 g honey	Diastatic index	Inverted sugar, %	Sucrose,%	Acidity mL 1N NaOH/100 g honey
Colza	2014	Max	19.8	1.76	38.5	80	3.1	2.2
		Min	17	0.11	17.9	75.5	1.15	1.2
		Average	18.05	0.61	25.51	77.68	2.13	1.75
	2015	Max	19.2	1.86	38.5	80.27	2.88	2.3
		Min	17	0.19	17.9	76	1.17	1.3
		average	17.96	0.76	27.05	78.12	2.00	1.75
	2016	Max	19.6	2.37	38.5	79.2	2.68	2.4
		Min	17.2	0.05	17.9	75.73	1.42	1.2
		Average	18.16	0.89	27.91	77.38	1.98	1.79
Acacia	2014	Max	18.6	4.4	23.8	75	4.75	1.9
		Min	15.3	0.19	13.8	70	2.17	0.8
		Average	16.83	0.79	18.67	72.89	3.20	1.16
	2015	Max	18.7	2.53	23.8	74.73	4.96	1.7
		Min	14.6	0.01	10.9	70.29	2.05	1
		Average	16.27	0.62	17.31	73.08	3.68	1.27
	2016	Max	20	3.11	23.8	75.73	4.95	1.9
		Min	14	0.09	10.9	70.55	1.67	0.9
		Average	16.77	0.65	17.22	73.50	3.76	1.23

Honey type	Year	Range	Water,%	HMF mg/100 g honey	Diastatic index	Inverted sugar, %	Sucrose,%	Acidity mL 1N NaOH/100 g honey
Linden	2014	Max	19.00	3.11	38.50	77.00	4.00	4.00
		Min	15.40	0.19	17.90	72.00	1.44	1.00
		Average	17.25	1.10	26.24	74.03	2.86	2.24
	2015	Max	19.00	2.76	38.50	79.20	4.75	3.50
		Min	16.20	0.03	17.90	70.23	1.15	1.20
		Average	17.47	0.61	25.39	75.02	2.44	2.25
	2016	Max	19.40	2.76	38.50	76.70	3.90	3.50
		Min	16.20	0.03	17.90	70.35	1.40	1.30
		Average	17.68	0.61	26.65	73.66	2.86	2.27
Polyfloral	2014	Max	19.80	4.37	50.00	80.95	3.97	4.00
		Min	14.60	0.11	17.90	71.73	1.17	1.40
		Average	16.96	1.18	31.89	76.94	2.48	2.83
	2015	Max	20.00	5.00	50.00	78.50	4.07	3.85
		Min	14.30	0.05	13.90	72.34	1.17	2.00
		Average	17.02	1.07	31.13	74.92	2.61	2.84
	2016	Max	20.00	4.39	50.00	79.23	4.27	3.90
		Min	14.50	0.19	13.90	72.50	1.42	1.20
		Average	16.64	1.32	30.24	75.93	2.76	2.65

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

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 fulfil the inverted sugar and sucrose requirements (**Table 1**).

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

Honey type		Sucrose	Inverted sugars	Diastatic index	HMF	Acidity	Water
Colza	F_{test}	1.05	3.91	1.23	2.61	0.90	0.57
	F_{crit}	3.10	3.10	3.10	3.10	3.10	3.10
	p Value	0.35	0.023	0.28	0.078	0.90	0.56
	Relevance	No	Yes	No	No	No	No
Acacia	F_{test}	2.98	1.52	1.39	0.36	2.19	2.43
	F_{crit}	3.10	3.10	3.10	3.10	3.10	3.10
	p Value	0.055	0.22	0.25	0.69	0.11	0.09
	Relevance	No	No	No	No	No	No
Linden	F_{test}	2.98	5.25	0.38	5.56	0.2	1.18
	F_{crit}	3.10	3.10	3.10	3.10	3.10	3.10
	p Value	0.055	0.007	0.67	0.005	0.90	0.28
	Relevance	No	Yes	No	Yes	No	No
Polyfloral	F_{test}	0.87	7.51	0.21	0.57	0.86	0.79
	F_{crit}	3.10	3.10	3.10	3.10	3.10	3.10
	p Value	0.41	0.0008	0.81	0.56	0.42	0.45
	Relevance	No	Yes	No	No	No	No

Table 2. One-way ANOVA results considering as factor the honey collection year.

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	Statistics	Colza	Acacia	Linden	Polyfloral
HMF, mg/100 g	Mean	0.75	0.69	0.78	1.17
	St. deviation	0.48	0.80	0.69	0.98
	Skewness	0.96	2.43	1.66	2.04
	Kurtosis	0.69	4.29	2.08	2.66
Acidity, mL 1 N NaOH/100 g	Mean	1.79	1.23	2.27	2.65
	St. deviation	0.31	0.25	0.60	0.68
	Skewness	0.12	1.34	0.42	0.07
	Kurtosis	−0.89	1.80	−0.58	−0.31
Diastatic index	Mean	26.82	17.73	26.09	31.04
	St. deviation	5.92	3.78	5.65	9.03
	Skewness	0.71	0.29	0.64	0.36
	Kurtosis	−0.07	−0.66	0.22	−0.10
Inverted sugar, %	Mean	77.73	73.16	74.24	75.95
	St. deviation	1.06	1.40	1.76	2.18
	Skewness	0.11	0.53	−0.39	0.38
	Kurtosis	−0.46	−0.60	0.09	−0.56
Sucrose, %	Mean	2.04	3.55	2.72	2.62
	St. deviation	0.42	0.97	0.78	0.81
	Skewness	0.25	0.10	−0.08	−0.07
	Kurtosis	−0.09	−1.30	−0.33	−0.90
Water, %	Mean	18.06	16.62	17.47	16.87
	St. deviation	0.69	1.09	0.82	1.21
	Skewness	0.45	0.57	0.02	0.29
	Kurtosis	−0.49	0.87	−0.28	0.46

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

pattern. 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.

Measured characteristic	Sugar	Inverted sugars	Diastatic index	HMF	Acidity	Water
F test value	58.20	132.23	68.64	7.33	45.71	38.88
F critical value	2.63	2.63	2.63	2.63	2.63	2.63
p value	1.2E-30	1.5E-57	4.8E-35	8.8E-05	1.4E-19	9.4E-22
Relevance	Yes	Yes	Yes	Yes	Yes	Yes

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

Principal component analysis, as 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

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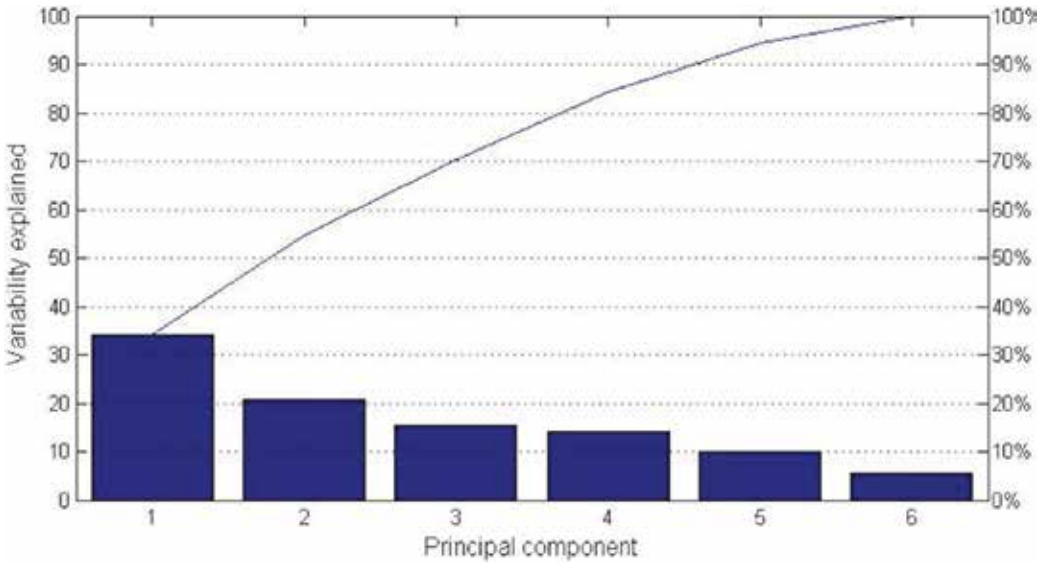

Figure 8. Principal component contribution in the data variability.

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**).

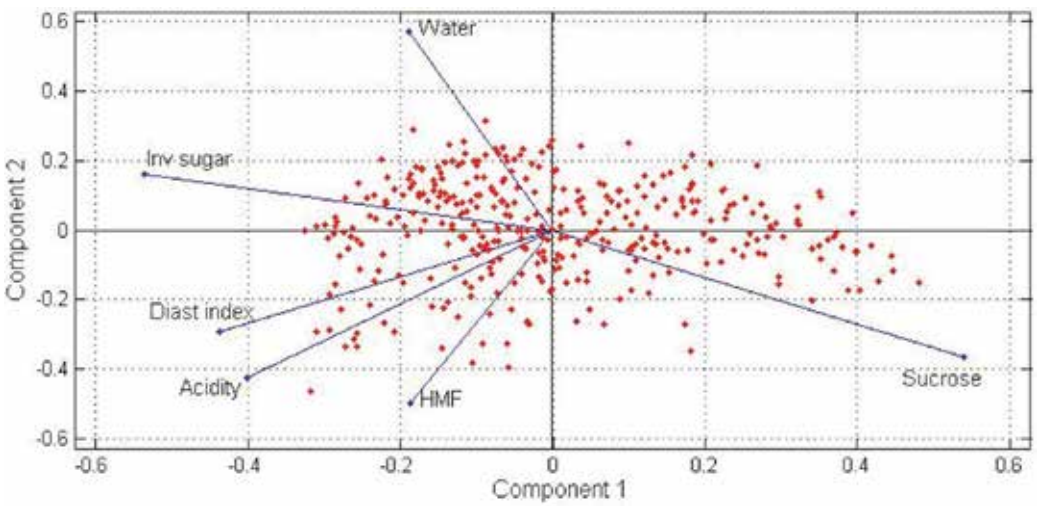

Figure 9. Bi-plot representation in the frame of principal component analysis.

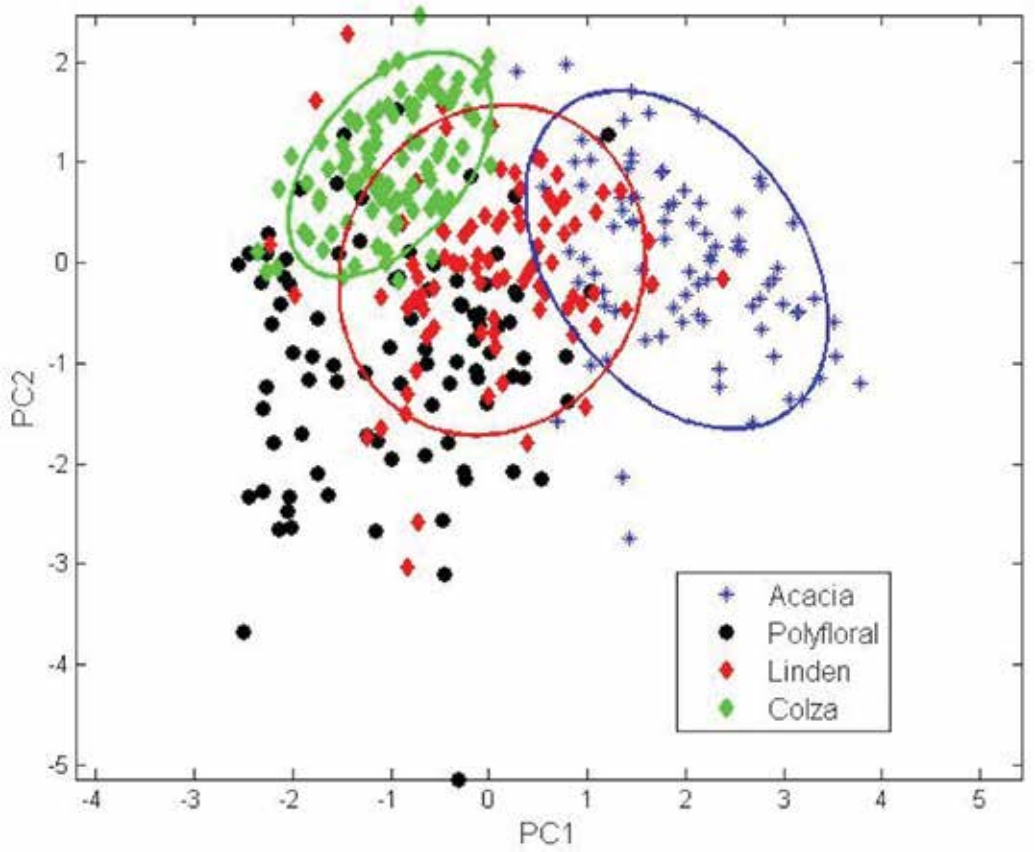

Figure 10. Data projection of four honey type samples in the principal components space.

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 analysis 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

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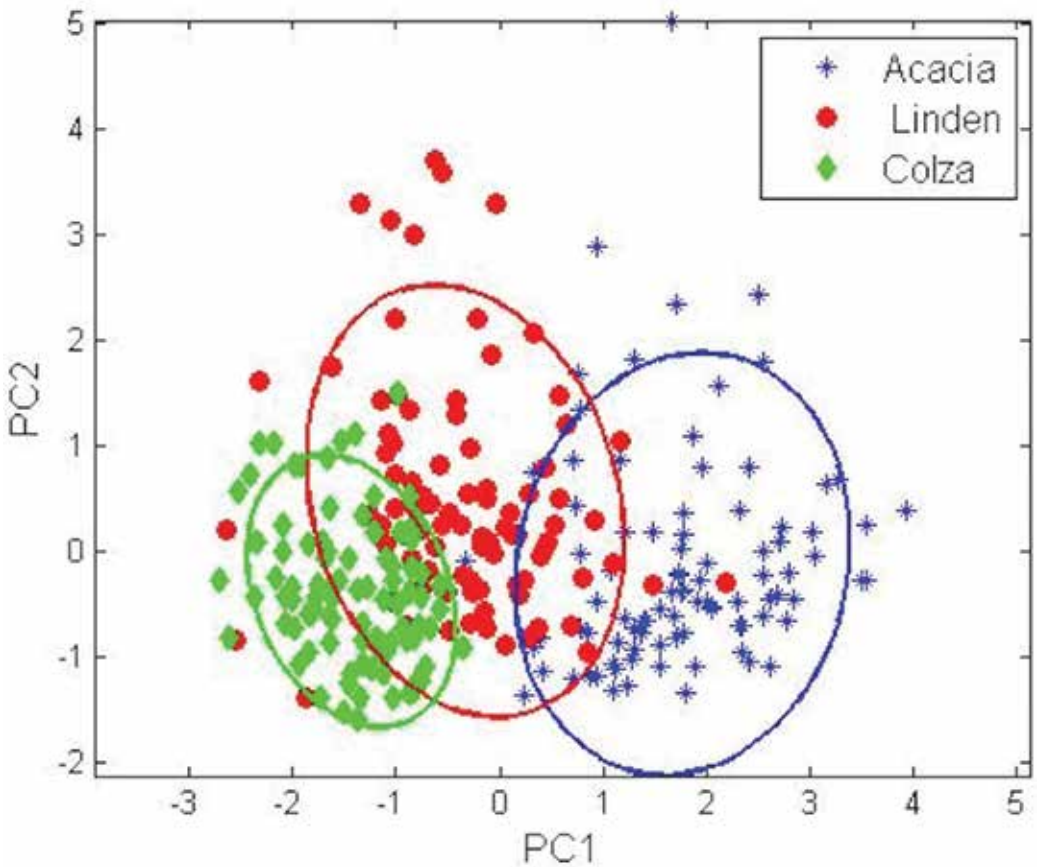

Figure 11. Data projection of unifloral honey samples in the PC1-PC2 space.

colza and acacia samples form distinct groups, approximately 30–40% of linden and polyfloral samples are miss-classified. 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.

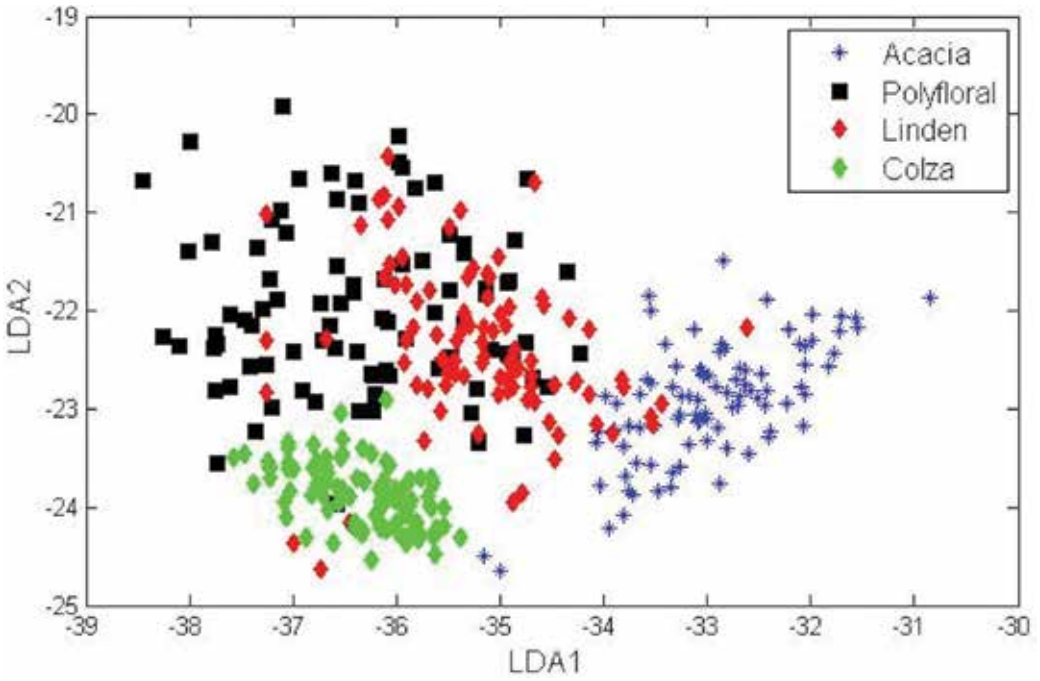

Figure 12. Data discrimination along the first and second linear discriminant analysis functions for the four honey type samples.

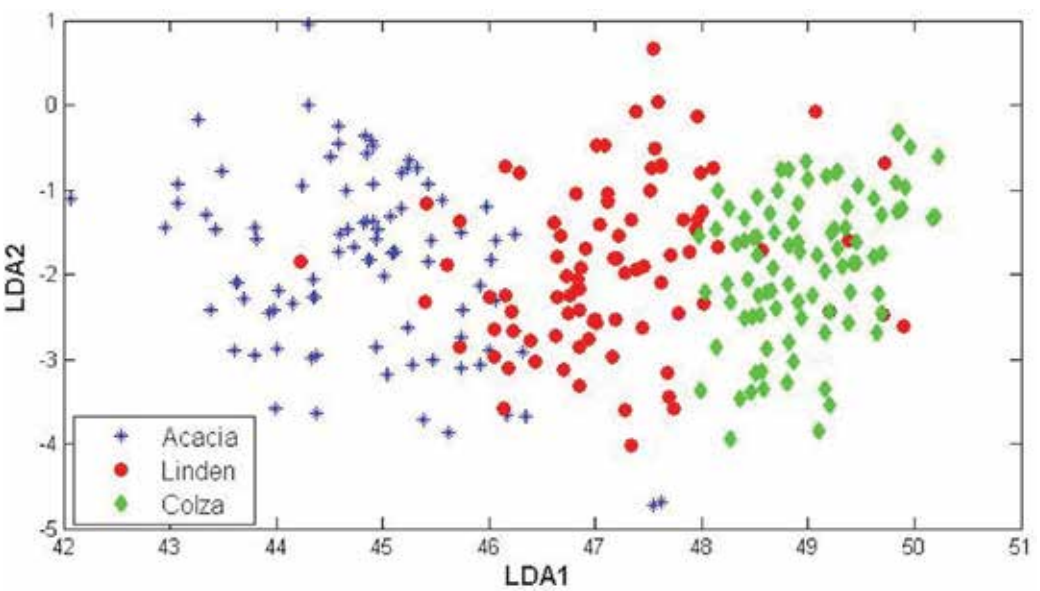

Figure 13. Data discrimination along the first and second linear discriminant analysis functions for unifloral honey samples.

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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.

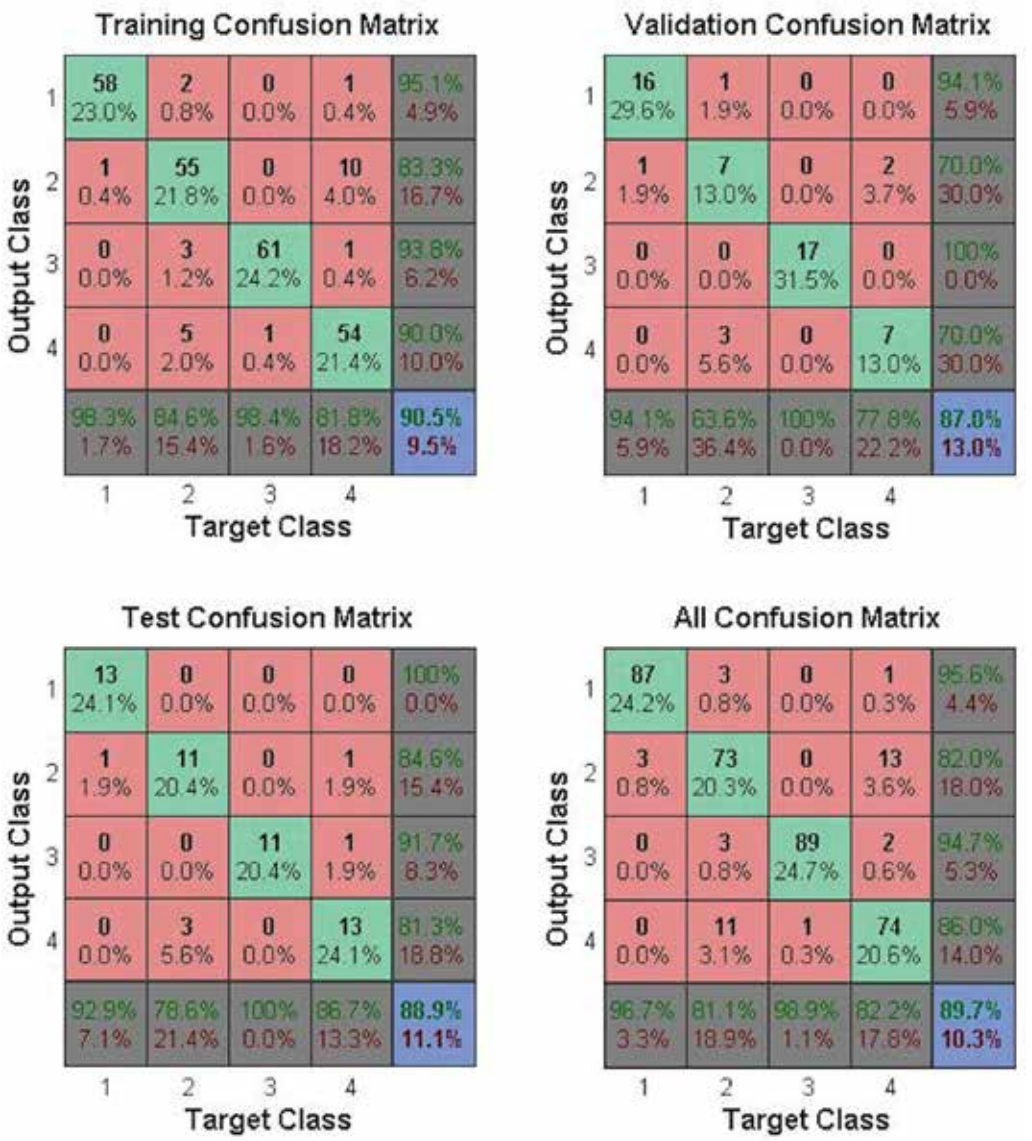

Figure 14. Confusion matrix for unifloral and polyfloral samples classification (1–acacia, 2–linden, 3–colza, 4–polyfloral).

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**).

Figure 15. Confusion matrix for unifloral samples classification (1–acacia, 2–linden, 3–colza).

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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Chapter 3

The Value of Chilean Honey: Floral Origin Related to their Antioxidant and Antibacterial activities

Raquel Bridi and Gloria Montenegro

Additional information is available at the end of the chapter

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Abstract

Honey chemical composition is related to the plant species where nectar is collected by honeybees. Chilean beekeeping is characterized by a variety of honey types, some unique, due to a high participation of endemic and native species. In Chile, the most emblematic flower honey, both for its abundance and sensory characteristics, is ulmo honey (*Eucriphya cordifolia*) and quillay honey (*Quillaja saponaria*). Melissopalynological analyses are used to establish whether a honey is unifloral, where at least 45% or more pollen grains found in it belong to the same species. The antioxidant and antimicrobial activities of Chilean honey have been studied in the last years with excellent results. *Quillaja saponaria, Eucriphya cordifolia, Azara petiolaris*, and *Retanilla trinervia* are within the Chilean endemic species that produce unifloral honeys that show antioxidant potential and antibacterial activity against pathogenic gram positive and gram -negative bacteria and also multiresistant strains. These activities are mainly attributed to the phenolic compounds such as flavonoids. Among these attractive characteristics of honey, it is important to note that this product has low toxicity and the medicinal properties of honey will help to protect honeybees by adding value not only to the significantly important process of pollinating crops and native plants, but also for the medicinal importance of their products.

Keywords: Chile, *Apis mellifera*, honey, phenolic compounds, biological activity, antioxidant, antibacterial

1. Introduction

In Chile, the natural and endemic flora offers many plants with invaluable potential biological properties that may be inherited for products originated from this flora such as honeybee products. Likewise, beekeeping is active and there are several unifloral and endemic Chilean honey that have been reported to have important biological properties such as ulmo

honey (originating from *Eucriphya cordifolia*), quillay honey (originating from *Quillaja saponaria*), tevo honey (originating from *Retanilla trinervis*), and others. The botanical origin of honey may be known through a quantitative and qualitative melissopalynological analysis. Honeybees are selective in the use of flower resources. The dominant plant community in Central Chile corresponds to the Matorral, an evergreen sclerophyllous vegetation with quillay and tebo as dominant plant species. The deep south of the country is dominated by temperate forest where ulmo is one of the dominant species. Biodiversity varies along an altitudinal or latitudinal gradient in Chile, so the beekeepers usually maintain their beehives along the native plant communities, so the bee products, as well as their potential biological properties will also be different depending on the botanical and geographical origin. Among the bioactive molecules inherited from a specific floral source, phenolic compounds obtained from honey have been related with the antioxidant and antibacterial properties that they show. Honey as a natural product offers many advantages that classify it as an excellent source of active molecules, which could be used as a treatment of human diseases in the forthcoming years. Among these attractive characteristics of honey, it is important to note that this product has low toxicity and the medicinal properties of honey will help to protect honeybees from disappearance by adding value not only to the significantly important process of pollinating crops and native plants, but also for the medicinal importance of their products.

2. Chile's unique geographical features and its endemic flora

The continental Chilean territory has an area close to 75 million hectares and is situated on the southwest border of South America. It has a length of approximately 4300 km from north to south and the average width is 180 km. Pits, terraces, mountainous regions, and valleys form Chile's diverse geomorphology, which together with the biogeographical isolation of a territory limited by geographical and climatic barriers, has configured a biodiversity characterized by a high level of endemism in ecosystems. Chile's vascular flora contains approximately between 5500 and 6000 species, without including subspecies and varieties. Although the number of species, compared with other South American countries, is not especially high, the most prominent trait of Chilean vascular flora is the presence of close to 50% endemic plant species, which gives the Chilean vascular flora a marked uniqueness [1]. Chilean apicultural production is defined by a high variety of honey types which contain a high percentage of nectar obtained from native plant species. The portion of nectar originating from native plants related to the endemism of Chilean flora result in the production of honey with unique characteristics.

The production of native and endemic monofloral honey is segregated into two large geographical areas: the first area corresponds to the central zone of Chile and the second corresponds to a region with a climatic transition from humid Mediterranean (VIII Region) to temperate humid (X Region). The central zone of Chile is of the five regions in the world that has a Mediterranean climate. It is characterized by a high level of endemism and biodiversity. Matorral is the dominant vegetal community in this zone. Characteristic matorral species include *Baccharis concave* (chilca), *Peumus boldus* (boldo), *Lithraea caustica* (litre),

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Trevoa trinervis (tevo), and *Q. saponaria* (soapbark tree). The central zone is characterized by the production of endemic monofloral honey from the quillay (*Q. saponaria*) and corontillo (*Escallonia pulverulenta*) species, while the southern zone, characterized by temperate forests, is characterized by native unifloral honey made from avellano *(Gevuina avellana)*, ulmo *(E. cordifolia)*, and tineo *(Weinmannia trichosperma)* [2].

3. Botanical origin of honey

Honeybees show great selectivity in the use of the vegetation surrounding their beehives. It has been shown that bees select plants with a high production of nectar, high concentration of sugar and that do not contain toxic compounds like certain alkaloids. Nevertheless, the presence of other secondary metabolites including terpenoids, phenolic acids, and flavonoids confer to honey import medicinal properties [3]. Nectar is an aqueous plant secretion whose content is mainly sugars and amino acids. It is collected by bees, particularly *Apis mellifera* L., and is converted into honey by enzymatic actions and dehydration, producing about 18% water content [4]. Honey is a food that contains about 200 substances and consists mainly of sugars, water, and other substances such as proteins (enzymes), organic acids, vitamins (especially vitamin B6, thiamine, niacin, riboflavin, and pantothenic acid), minerals (including calcium, copper, iron, magnesium, manganese, phosphorus, potassium, sodium, and zinc), pigments, solid particles derived from honey harvesting, a large variety of volatile compounds, and also secondary metabolites characteristic of the origin species like phenolic compounds and terpenes [4, 5].

Melissopalynology is the division of palynology, which studies the botanical and geographical origin of honey by subjecting honey sediment, and therefore pollen grain and the other structures therein, to microscopic analysis (**Figure 1**). Honey pollen profiles indicate floral diversity, forest vegetation, and species composition of plants that honeybees forage. The relative pollen frequency is utilized for tagging purposes and to ensure geographical origin, factors which considerably influence honey's commercial value. Furthermore, relative pollen frequency is also utilized as a traceability tool by food control institutions and to assess correlations with *in situ* climatic parameters such as rainfall and temperature, important external factors influencing pollinators and pollination networks [6, 7]. In Chile, the official policy (NCh2981.Of2005) established by the Standards Division of the National Institute for Standardization [2] indicates that the melissopalynological test must be used to differentiate the botanical origin of honey produced in this country. In agreement with this regulation, honey can be classified according to three types of botanical origins: monofloral, bifloral, or polyfloral. Monofloral or unifloral honeys are those where at least 45% or more pollen grains found in it belong to the same species; bifloral honeys are those where pollens from two species are dominant within the total pollen grains, so that, as a whole, both species cover more than 50% of the total pollen grains, and there is not a difference higher than 5% among them; and finally, polyfloral honeys are those where none of the requirements for monofloral and bifloral honeys are met, that is, those where no species reaches at least 45% of the total pollen grains, nor two of them covers more than 50% of the said total.

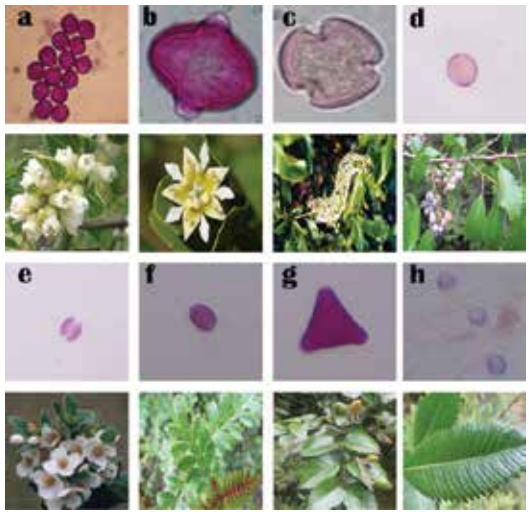

Figure 1. Plants species and respective pollens (microscope 400x) (a) *Retanilla trinervia* (tevo), (b) *Quillaja saponaria* (quillay), (c) *Escallonia pulverulenta* (corontillo), (d) *Azara petiolaris* (corcolén), (e) *Eucryphia cordifolia* (ulmo), (f) *Weinmannia trichosperma* (tineo), (g) *Gevuina avellana* (avellano chileno), and (h) *Caldcluvia paniculata* (tiaca).

The melissopalynology technique is quite laborious, time-consuming and requires a high-skilled and trained technician. Thus, a large number of research groups worldwide have focused their attention and studies on improving the knowledge of honey characterization. The most promising approach appears to be the simultaneous detection of multiple components utilizing spectroscopic methods along with statistical analysis. Chemometrics along with Raman, FTIR, NMR, and NIR spectroscopic methods have been used for defining the floral origin of honey and development of classification models. These procedures promptly provide quantitative information without complex pretreatment of samples and primarily through a single spectroscopic technique [6, 8, 9]. Despite advances in these methods in the last few years, limitations still exist in these studies related to the small number of samples and the validity of the proposed methods are rarely demonstrated [8]. Notwithstanding, the emerging new methods are making way to new frontiers in honey characterization. The most promising strategy appears to be the multidisciplinary one, which focuses on the detection of multiple components assisted by chemometrics. Apicultural industries and small producers will make the most of the advantages of more advanced methods which allow for more scrupulous controls, increasing the quality level and safety of honey and derivatives [9].

4. Chilean unifloral honey

Chile produces a limited number of unifloral honeys with native plant origins. Montenegro et al. [10] identified the species of native plants that *A. mellifera* uses as the most intensive

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source of nectar. These species include *Q. saponaria* (quillay, soapbark), *E. cordifolia* (ulmo), *G. avellana* (avellano), *E. pulverulenta* (corontillo), *R. trinervia* (tevo), *Caldcluvia paniculata* (tiaca), *W. trichosperma* (tineo), and species of genus *Azara* (corcólen). They are used as the source for monofloral honey, which are selected by honeybees mainly due to the volume and chemical composition of nectar offered by the flowers [4, 7, 10] (**Table 1**).

Origin	Common name	Plant species	Family
Zone			
Forest temperate (Southern Chile)	ulmo	*Eucryphia cordifolia* Cav.	Cunoniaceae
	tiaca	*Caldcluvia paniculata* (Cav.) D. Don	Cunoniaceae
	tineo	*Weinmannia trichosperma* Cav.	Cunoniaceae
	avellano	*Gevuina avellana* Molina	Proteaceae
Matorral (Central Chile)	quillay	*Quillaja saponaria* Monlina	Quillajaceae
	tevo	*Retanilla trinervia* (Gillies & Hook.) Hook. & Arn.	Rhamnaceae
	corontillo	*Escallonia pulverulenta* (Ruiz & Pav.) Pers.	Escalloniaceae
	corcolen	*Azara petiolaris* (D. Don) I.M. Johnst.	Salicaceae

Table 1. Botanical origin of unifloral honey in Chile.

A recent study of the biological properties of Chilean unifloral honeys indicates that Chilean native honey presented significant differences in their antioxidant as well as biological activity, which depends on the botanical and geographical origin, and can be associated with polyphenol content. Moreover, the presence of other species in the total botanical content of honey plays an important role in the modulation of its biological properties [11].

In Chile, the most emblematic flower honey, both for its abundance and sensory characteristics, is quillay (*Q. saponaria)* and ulmo (*E. cordifolia*). The antioxidant and antimicrobial activities of Chilean honey have been studied in the last years with excellent results. *Q. saponaria, E. cordifolia*, and *R. trinervia* are within the Chilean endemic species that produce monofloral honey that show antibacterial activity against pathogenic Gram-positive and Gram-negative bacteria and also multiresistant strains [4, 10–14]. With regard to antioxidant activity, honey from *Q. saponaria* and *Azara petiolaris* stand out due to potential shown in various *in vitro* models utilized to evaluate natural antioxidant capacity to inactivate reactive species. The positive correlation between phenolic compounds and antioxidant capacity is verified in some of these models. Phenolic compounds such as aromatic acids and flavonoids are considered to be responsible for antioxidant capacity since they have a chemical structure particularly suitable to exert an antioxidant action acting as free radical scavengers neutralizing reactive oxygen species and chelating metal ions.

5. Phenolic compounds

Phenolic compounds are plant-derived secondary metabolites, biosynthesized mainly for protection against stress and oxidative damage and transferred via the nectar to the honey. The intensity of the color of a honey may be associated with the antioxidant strength of the honey. The phenolic, flavonoid, and carotenoid content is increased in darker honeys and reduced in lighter more transparent honey. As a result, biological properties, such as antioxidant activities and antibacterial capabilities, of the honey are related to its color, and darker honey tends to have enhanced properties [4, 15].

The most common phenolic compounds are phenolic acids and flavonoids [16]. Phenolic acids constitute an important class of phenolic compounds with bioactive functions typically found in vegetable products and foods. Also are secondary metabolites required for normal operation of naturally occurring plants. They can be divided into two subgroups according to their structure: the hydroxybenzoic and hydroxycinnamic acids. Acids derived from hydroxybenzoic acids include þ-hydroxybenzoic, vanillic, syringic, salicylic (2-hydroxybenzoate), gallic, and ellagic. These compounds might be existing in soluble form in cells, along with sugars or organic acids, or formed with cells linked to lignins. Hydroxycinnamic acids occur normally in their conjugated form as esters of hydroxy acids such as tartaric acid and shikimic as well as in their pure form, including þ-coumaric, caffeic, ferulic, and sinapic acids. Flavonoids (flavones, flavonols, flavanones, flavanols, anthocyanidin, isoflavones and chalcones) are the largest group of plant phenolic compounds. This group represents over 50% of all naturally occurring phenolic compounds. They are generally distributed in the seeds, bark, leaves, and flowers of plants and trees. In plants, these compounds give protection, against pathogens, herbivores, and UV radiation [5, 17].

The qualitative and quantitative difference in the phenolic profile of honey according to the different botanical sources represents the scientific basis of the two main lines of research about the study of honey phenolic fraction. The first approach is focused on the evaluation of the bioactive properties of honeys while the second approach attempts to attribute the botanical and/or the geographical origin of honey based on the existence and the abundance of at least one or more specific phenolic compounds, thus proposed as chemical marker(s) of origin. The results of these research studies are relevant in both directions; honey of varying botanical origins show a wide range of health-promoting properties like antibacterial, antiinflammatory, antioxidant, and radical-scavenging activity [4, 16, 18]. A wide range of phenolic constituents are present in honey such as quercetin, caffeic acid, caffeic acid phenethyl ester (CAPE), acacetin, kaempferol, galangin, chrysin, pinocembrin, pinobanksin, and apigenin, which have promising effects in the treatment of some diseases [19, 20].

Pinocembrin, pinobanksin, and chrysin are the characteristic flavonoids of propolis and these flavonoids have been found in European [19, 21] and Chilean honey samples [22]. Small amounts of propolis might be incorporated into honey; therefore, propolis flavonoids might contribute to the phenolic composition of honey. In temperate areas, the main sources of propolis are poplar (*Populus*) bud exudates. The identification of propolis-derived compounds like pinocembrin and chrysin could have an important contribution to the phenolic composition and antioxidant activity in corcolen (*Azara petiolaris*) Chilean honey [22].

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Antioxidant, antiinflammatory, antimicrobial, antiviral, and antiulcerous action, and the capability for regulating enzymatic browning are some of the principal characteristics of honey primarily attributed to phenolic compounds. The implementation of honey polyphenols has recently gained great interest from the functional food, nutraceutical and pharmaceutical industries. However, the efficacy of polyphenols relies on the preservation of their stability and bioactivity. Phenolic compounds, as well as other organic compounds, are degraded depending on the environmental conditions to which they are subjected. Spanish honey samples were subjected to liquefaction and liquefaction/pasteurization and the phenolic compounds evaluated as to the impact of industrial heat treatment. Phenolic compounds found in these honey samples were caffeic and þ-coumaric acids and flavonoids naringenin, hesperetin, pinocembrin, chrysin, galangin, quercetin, and kaempferol. A significant decrease in the concentration of galangin, kaempferol, myricetin, and þ-coumaric acid was observed after heat treatment [23]. Moreover, some flavonoid glycosides present in honey demonstrate certain instability under slight alkaline conditions and high sensitivity to oxidation in the presence of slight oxidizing agents such as hydrogen peroxide, which is present in honey and is responsible for the degradation verified in the flavonoids analyzed [24, 25].

The complexity of a food matrix like honey implies that the target analytes are usually present in low concentrations, and this demands the adoption of a multistep analytical procedure able to provide a careful measurement of these quantities [16]. Procedures using Amberlite XAD-2 columns for cleaning the complex matrices of honey and isolation of their phenols are often performed. In some cases, this step would reduce the need for sample manipulation and give a sample extract uniformly enriched in all components of interest and free from interfering matrix components. In these procedures, aqueous- acidified honey solutions are passed through the columns to retain phenols in sorbent beds and afterward eluded with methanol [19, 26]. These extracts are widely employed in analytical methods, biological assays, and functional food development, since the presence of sugars gives the entire honey a syrupy texture, which causes difficulties for some analysis and preparations. However, recoveries of phenolic acids and flavonoids extracted from deionized water (pH 2) using Amberlite XAD-2 demonstrated different recovery percentages, probably depending on the structure of the phenols studied. Kaempferol, *p*-coumaric acid, and syringic acid were completely adsorbed, but the recovery of gallic acid, caffeic acid, and quercetin by methanol is much less efficient [13, 27–29].

6. Abscisic acid

Abscisic acid, a plant hormone related to the protection of plants in environmental stress conditions, has been detected in corcolen and quillay honeys. The existence of abscisic acid in nectar is well established and is affected by environmental conditions, which might regulate the biosynthesis of certain secondary metabolites, such as phenolic compounds and abscisic acid. The biosynthesis of these compounds may be stimulated by plants, lowering damages through their capacity to capture free radicals under stress conditions, and reduce the penetration of UV-B ultraviolet radiation. The representation of these compounds in honey produced from *Q. saponaria* may be associated with the high interannual variability of climate conditions of the central zone of Chile [13, 30].

7. Biological activities

Clinical investigations of the therapeutic potential of honey are gradually growing and scientific evidence for the efficacy of honey in some conditions is beginning to emerge. The healing effect of honey could be classified by its antiinflammatory, antibacterial, and antioxidant properties of its components. Furthermore, honey has been reported to be effective in gastrointestinal disorders, in healing of wounds and burns, and in treating venous ulcers [31].

8. Antioxidant activity

Over many years, honey from different parts of the world have been shown to be one of the highest potential natural products in which phenolics, flavonoids, ascorbic acids, and some enzymes serve as potent antioxidants [32]. The antioxidant properties of honey are derived from both enzymatic (e.g., catalase, glucose oxidase, and peroxidase) and nonenzymatic substances (e.g., phenolic compounds, ascorbic acid, α-tocopherol, carotenoids, amino acids, proteins, and Maillard reaction products). The quantity and kind of these antioxidants are mainly dependent on the floral source. The main functional components of honey are flavonoids. They contribute significantly to the total antioxidant activity of honey and they act by several mechanisms including direct trapping of reactive oxygen species, inhibition of enzymes responsible for producing superoxide anions, chelation of transition metals involved in processes forming radicals, and prevention of the peroxidation process by reducing alkoxyl and peroxyl radicals [5, 15, 29]. The antioxidant activity of flavonoids in the majority of cases relies on the number and position of hydroxyl groups, additional substituents, and the glycosylation of flavonoid molecules. The presence of specific hydroxyl groups in the flavonoid rings improves antioxidant activity. Substitution patterns in the A ring and B ring, and the 2,3-double bond (unsaturated) and 4-oxo group in the C ring affect the antioxidant action of flavonoids as well. The glycosylation of flavonoids reduces their antioxidant activity compared to the analogous aglycones [5, 33].

These antioxidants may help to protect cellular damages from oxidative stress and lower the risk of chronic diseases. Furthermore, in recent years, there has been an increase in new methods for the research of free radicals and antioxidants in relation with advances in human health. Various studies have demonstrated that neuronal and behavioral changes occur with ageing, including in the absence of degenerative disease. Current studies indicate that dietary intake of antioxidant nutrients and cognition is closely related. Evidence from epidemiological, experimental and clinical studies demonstrates that the consumption of foods with high levels of dietary antioxidants might prevent or lower the risk of cognitive deterioration [34]. Many research models have been established in chemical and/or biological systems for the studies of mechanisms of action of antioxidants. Generally, antioxidant ability was measured and presented as total antioxidant capacity (TAC) [35, 36], total antioxidant potentials (TRAP) [37, 38], Trolox equivalent antioxidant capacity (TEAC) [39], ferric reducing/antioxidant power (FRAP) [40], and oxygen radical absorption capacity (ORAC) [41]. Mechanistically, these methods are based on either a single-electron transfer reaction or a hydrogen atom

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transfer reaction from an antioxidant or oxidant to a free radical. The total antioxidant activity is related to the radical scavenging ability and reductive activity [42].

Montenegro et al. [11] studied the antioxidant activity of unifloral honeys (quillay, ulmo, avellana, tiaca) of native plants from Chile. In this study, was observed an important correlation between total phenolic content and antioxidant activity evaluated by ferric reducing activity power—FRAP method. The ferric reducing activity power assay directly measures antioxidants with a reduction potential below the reduction potential of the Fe^{3+}/Fe^{2+} couple and the reaction is reproducible and linearly related to the molar concentration of the antioxidant(s) present in the sample. Furthermore, some variations in antioxidant activity between honey samples with the same botanical origin were observed. This variability could be explained by different accompanying species and geographical origin zone of the honey [11].

The scavenging activity towards peroxyl/alkoxyl radicals (ORAC method) is one of the most employed assays. In fact, in the last years databases of the ORAC index of foods have been built to emphasize the benefits of establishing the antioxidant capacity of polyphenol-rich foods [43]. The method is based on the ability of antioxidants to prevent the consumption of a target molecule mediated by free radicals generated during the aerobic thermal decomposition of AAPH (2,2′-azo-bis(2-amidinopropane). The target molecules are most commonly used are beta-phycoerythrin, fluorescein, and pyrogallol red. The use of the pyrogallol red (PGR) as probe is related to the amount and reactivity of a given phenolic compound towards the free radicals generated in the AAPH (2,2′-azo-bis(2-amidinopropane) dihydrochloride) thermolysis. The ORAC-PGR index can be considered as a measure of the capacity of the sample to remove peroxyl and alkoxyl radicals [44]. In complex mixtures, concentration, chemical nature, and possibly the interaction between the antioxidants present in the sample determine this index.

Recently obtained results from our research group demonstrated that quillay honey's ORAC-PGR index is not correlated to phenolic compound content present in samples but is highly correlated to flavonoid content. This is due to the fact that flavonoids are the polyphenolic species to which the highest reactivity is attributed [45]. In addition, the ORAC-PGR index in honey of *A. petiolaris*, named commonly as corcolen honey, collected from honeybee colonies of the central zone of Chile was evaluated. The value of this parameter for these samples is correlated to the percentage of corcolen pollen present, meaning that mostly the phenolic compounds and compounds with scavenger capacity belong to corcolen species. These correlations are quite interesting since just by means of the melissopalynological assay there could be evidence of the phenolics composition as well as the antioxidant capacity of monofloral *Azara sp*. honey [22].

Finally, more recently, our group has obtained results indicating that honey quillay compounds are reactive toward hypochlorite (HOCl). Hypochlorite has an important role in defense mechanisms that take part in the immune response toward microorganisms. However, it has also documented that hypochlorite, in certain pathophysiological conditions, can damage macromolecules including proteins, DNA, RNA, and cell membrane lipids, changing their biological function. The consumption of PGR-induced by hypochlorite is inhibited by compounds able to react with this reactive species. PGR-hypochlorite indexes obtained for quillay honey samples indicated high hypochlorite-mediated oxidation protection potential, these results being comparable to those obtained via Trolox—water-soluble vitamin E analogue (unpublished results).

9. Antibacterial activity

The broad-spectrum of antimicrobial activity of honey was demonstrated in various studies and reportedly exerts both bacteriostatic and bactericidal activities. The antimicrobial nature of honey depends on different factors acting singularly or synergistically, the most significant of which are phenolic compounds, pH of honey, H_2O_2, wound pH, and osmotic pressure exerted by the honey itself [15, 46]. The antibacterial capabilities of different unifloral Chilean honey, including ulmo honey (*E. cordifolia*), quillay honey (*Q. saponaria*), avellano honey (*G. avellana*), and tiaca honey (*C. paniculata*) were analyzed (**Table 2**). The methanolic extract of these honeys, obtained using Amberlite XAD-2 column, demonstrated better antibacterial capabilities than the honeys themselves, indicating an important role of the phenolic compounds in this activity. In *in vitro* assays, all of the honey extracts were able to inhibit the growth of *Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus*, and *Streptococcus pyogenes* determining minimal bactericidal concentration [4, 11].

Floral Origin	Antioxidant/Antibacterial activities	Reference
Quillay honey (phenolic extracts)	Antibacterial activity against *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus typhi, S. aureus, Streptococcus pneumoniae, Vibri cholerae* and antifungal activity against *Candida albicans*.	Montenegro et al. [11]
Quillay honey (entire honey and phenolic extracts)	Oxygen radical absorbance capacity (ORAC-PGR) index related to the capacity of the sample to remove peroxyl and alkoxyl radicals	Bridi et al. [45]
Ulmo honey	Comparison of the antimicrobial activity of ulmo and manuka honey against methicillin-resistant *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*.	Sherlock, et al. [14]
Ulmo honey (phenolic extracts)	Inhibition of *in vitro* growth of human pathogenic bacteria *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and fungicidal activity on fungi genera *Mucor, Rhizopus, Aspergillus, Candida* and *Penicillium*	Montenegro and Ortega [48].
Ulmo honey	Ulmo honey topical application supplemented with ascorbic acid improves regeneration in burns in guinea pig.	Schencke, et al. [49–51]
Ulmo honey	Clinical trial: topical treatment using ulmo honey associated with oral ascorbic acid showed excellent clinical results for the healing of venous ulcers	Calderon et al. [52]
Ulmo, quillay avellana and tiaca honeys	Ferric reducing antioxidant power	Montenegro et al. [11]
Corcolen honey	Oxygen radical absorbance capacity (ORAC-PGR), index related to the capacity of the sample to remove peroxyl and alkoxyl radicals	Giordano et al. [22]

Table 2. Review of antioxidant and antibacterial activity in unifloral Chilean honey.

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The manuka honey derived from the manuka tree (*Leptospermum scoparium)*, which grows as a shrub, or a small tree throughout New Zealand and eastern Australia is the best known of the honeys. It has been reported to have an inhibitory effect on around sixty species of bacteria, including aerobes and anaerobes, positives, and Gram-negatives. The antimicrobial activity exhibited against pathogenic bacteria such as *S. aureus* make this honey a promising functional food for the treatment of wounds. The potential of honey to assist with wound healing has been demonstrated repeatedly and the healing properties can be ascribed to the fact that it offers antibacterial activity, maintains a moist wound environment that promotes healing, and has a high viscosity that helps to provide a protective barrier to prevent infection [47]. A study compared the antimicrobial activity of the ulmo honey with manuka honey against five strains of methicillin-resistant *S. aureus, E. coli*, and *P. aeruginosa*. The ulmo honey had greater antibacterial activity against all methicillin-resistant *S. aureus* isolates tested than manuka honey and similar activity against *E. coli* and *P. aeruginosa* using agar diffusion assay. The minimum inhibitory concentration assay showed that a lower minimum inhibitory concentration was observed with ulmo honey than with manuka honey for all five methicillin-resistant *S. aureus* isolates. For the *E. coli* and *Pseudomonas* strains, equivalent minimum inhibitory concentration was observed. Due to its high antimicrobial activity, ulmo honey may warrant further investigation as a possible alternative therapy for wound healing [14]. In Chile, ulmo honey extract has been patented for its bactericidal and fungicidal properties [48]. The document relates to uses of an extract of unifloral ulmo honey, rich in phenolic compounds, able to inhibit the *in vitro* growth of human pathogenic bacteria such as *S. aureus, P. aeruginosa, and E. coli*, in addition to exhibiting fungicidal and fungistatic activity on fungi genera *Mucor, Rhizopus, Aspergillus, Candida*, and *Penicillium*.

The use of ulmo honey in association with oral vitamin C as an alternative in healing treatment of *burn wounds* in guinea pigs (*Cavia porcellus*) improves regeneration in this type of wound and also reduces the possibility of infection, inflammation, and edema [49–51]. In addition, the clinical effect of topical treatment with ulmo honey associated with oral vitamin C in patients with venous ulcers was evaluated. This treatment method presented significant results, healing wounds faster in 100% of patients with all types of venous ulcers. Furthermore, the honey presented nonadherent and debriding properties was straightforward to apply and remove, and was well received by users [52].

Regarding honey of *Q. saponaria*, the antibacterial and antifungal activities were analyzed. Extracts of unifloral honeys of quillay were tested for antibacterial activity on *P. aeruginosa, E. coli, Staphylococcus typhi, S. aureus, Streptococcus pneumoniae* type β, and *Vibrio cholerae*, and antifungal activity against *Candida albicans*. The best *in vitro* activity of these extracts were on *S. aureus* and hemolytic *S. β*, both of which affect the skin [13]. The antibacterial effects exhibited could be related to an overall effect of the phenolic compounds present in the extract (caffeic, coumaric and salicylic acids, the flavanone naringenin and the flavonol kaempferol), which were detected by high-performance liquid chromatography.

Microbial resistance to honey has never been reported which makes it a very promising topical antimicrobial agent against the infection of antibiotic-resistant bacteria and in the treatment of chronic wound infections that do not respond to antibiotic therapy. The potency of honey, such as Chilean honey, against microorganisms suggests its potential to be used as an alternative therapeutic agent in certain medical conditions, particularly wound infection.

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Chapter 4

Analysis of Amino Acid and Phenolic Content in Honey by UPLC-ESI-MS/MS

Şeyda Kıvrak

Additional information is available at the end of the chapter

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Abstract

Honey is the very valuable natural animal product. It offers more than hundred nutritional substances to its consumers, human being and animals. Though major constituent of honey is sugar and water, honey also possesses amino acids, phenolic compounds, vitamins, minerals and enzymes. Amino acids are one of the important components of food. They provide the required building blocks and protein synthesis. Moreover, phenolic compounds in honey constitute the important quality parameter and account for its colour, sensory properties and antioxidant activity. Analysis of phenolic compound and amino acid is very important. They are generally used to identify the origin of honey. Amino acids in honey come from animals and vegetables. In the literature, there are several techniques concerning amino acid and phenolic compound identifications. In this chapter, usage of ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry (UPLC-ESI-MS/MS) techniques and methods for the determination of amino acids and phenolic compounds of honey is explained.

Keywords: honey, amino acids, phenolic compounds, liquid chromatography, UPLC-ESI-MS/MS

1. Introduction

Honey is highly well known and one of the earliest animal products. It has been appreciated throughout civilization and has been recently produced widely in the world [1]. Honey is yellowish or brownish viscid fluid produced by honeybees from the nectar of flowers or from the secretion obtained from the living parts of plants [2]. The honey can be made from a variety of different flowers, and its flavour, texture and chemical composition depend on the floral source from which it was collected. They store the nectar in their sac and enrich it

with some of their own substances, invertase enzyme, to introduce chemical changes. It is produced naturally, and when the honeybees return to the hive, they deposit the nectar in honeycombs for storage and ripening [2, 3]. Honey offered to people since the ancient times. It has been used as medicinal food and preservative having multiple tastes and flavours. In addition, the honeybees do not hesitate to use the needle sometimes at the expense of their own lives and that needle is a drug given to human suffering, even natural therapeutic. Besides being enjoyed as honey, it is used in baking or manufacturing of alcoholic beverages by mixing alcohol or by fermentation into honey-flavoured wine. Preparations containing honey, in combination with milk and cereals, are processed for children. Tobacco products are occasionally flavoured with honey. In medicine, honey is used in pure form or prescribed in preparations such as honey milk, fennel honey and ointments for wounds. It is used in cosmetics as glycerol-honey gels and tanning cream products [4].

Honey includes more than hundred substances. The major constituent of honey is sugar and water; however, amino acids, phenolic compounds, vitamins, minerals and enzymes are found in it [5, 6]. In addition, they directly contribute to the flavour of food and precursors of aroma compounds and formed during thermal or enzymatic reactions in production, processing and storage of food. Honey proteins are derived partly from plants and partly from honeybees.

Amino acids are the building blocks of our body. Amino acids help cells regenerate. Regular nutrition helps us avoid getting older as long as human take the necessary amino acids. It is usually very effective in the formation and progression of muscle tissue. And human-being owe to amino acids for healthy nails and hair. Regulation of brain function, balancing the mental health, muscle making and energy are related to the function of amino acids. Moreover, in the treatment of several illnesses amino acids are used regularly. Obesity is known as the most common diseases of today, and amino acids are used for fat-shattering in situations where obesity and weight control are required. Amino acids also help to treat cerebral diseases such as attention-deficit hyperactivity, dementia, Alzheimer's, Parkinson's, though treatment of those diseases is not fully possible. But amino acid supplements help keep the disease under control. Amino acids are also used in the mental development of school children and in particular stress control from exams. Amino acids are used effectively during the post-operative healing process, especially in burn and wound treatments. Repair and renewal of the tissues is accelerated by amino acids. Another area of use is to delay skin ageing effects and wrinkles, to regain the elastic structure of the drying skin and to prevent hair loss.

As the amino acids are important components of food, and supply the required building blocks or protein synthesis. Considering honey, the amino acids come from animals and vegetables. Amino acid analysis is very important and generally used to identify the origin of honey. There are several techniques concerning amino acid identification, which include multiple steps: sample extraction procedure, derivatization of amino acids, separation and confirmation/quantitation; and also gas chromatography [7] and liquid chromatography [8, 9] methods have been used. Most of the published studies on the determination of amino acids in honey have used derivatization agents and solid-phase extraction (SPE). Although chromatographic

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separation of amino acids in honey has been confirmed in the literature, due to derivatization step, peaks appearing in residues and matrixes are still challenging, and frequently complete separation cannot be succeeded. It is considered that mass spectrometric (MS) detection is more selective than ultraviolet-visible (UV-Vis) or fluorescence [10]. It is very substantial to pay special attention to use of MS detection methods for amino acid analysis [1].

Phenolic compounds constitute the important quality parameter of honey and account for their colour, sensory properties and antioxidant activity. Phenolic compounds present in honey can be used as indicators of floral origins and botanical resources, such as pollens, nectars, resins and oils, and the quality of honey [2]. In addition, relatively little is known about honey colour pigments. As an example, the amber colour appears to originate from phenolic compounds and from products of non-enzymatic browning reactions between amino acids and fructose of honey. The health implications also warrant further knowledge of flavonoid contents of the food supply such as honey [13]. It is known that flavonoids and phenolic acids of honey are responsible for significant antioxidant capacity, and other beneficial pharmacologic properties of honey include wound healing, anti-inflammatory, anti-mutagenic and anti-tumoural, protection of skin cells and tissues from oxidative damage and food preservation. Therefore, it is highly demanding to analyse honey and find out which polyphenols are present and in what amount [2, 11, 12, 14, 15].

In this chapter, usage of ultra-performance liquid chromatography with electrospray ionization coupled to tandem mass spectrometry (UPLC-ESI-MS/MS) techniques and methods for the determination of amino acids and phenolic compounds of honey will be explained. In the eighteenth and nineteenth centuries, extremely important advances were made in the development of qualitative and quantitative methods for analysing organic substances. Hence, to check the authenticity and quality control of honey, it is necessary to establish a simple, fast and accurate method to perform extensive honey compositional analysis that will help to identify its most characteristic constituents.

2. Methods

2.1. Instrumental conditions of UPLC–ESI–MS/MS for the amino acid analysis and the phenolic compound analysis

The free amino acids and the phenolic compounds are identified in honey. The amino acid analysis method [1, 12, 16] and the phenolic compound analysis method [2, 11, 12] are easy, fast and reliable procedures without sample clean-up and without derivatization steps. The analyses were performed using an UPLC–ESI–MS/MS instrument, consisting of ultra-performance liquid chromatography with a column manager and heater/cooler, binary system manager, sample manager coupled to a triple quadrupole mass spectrometer equipped with electro spray ionization (ESI). The mass spectrometry parameters, confirmation and quantification mass transition (m/z), and their collision energies are listed in **Tables 1** and **2** for amino acids and phenolic compounds, respectively. Separation operations are accomplished using a C_{18} column, and gradient mobile phase conditions are given in **Tables 3** and **4**, respectively.

Amino acid	Retention time (min)	Quantification transition (m/z)	Confirmatory transition (m/z)	Collision energies (V)
Glycine	0.58	76.00	30, 44, 76	8, 8, 3
Alanine	0.59	90.00	57.1, 71	8, 8
Serine	0.58	106.00	60, 88	9, 10
Proline	0.67	116.10	43.3, 70,1	22, 12
Valine	0.83	118.10	55, 72	18, 10
Threonine	0.61	120.10	56.1, 74, 84, 102.1	15, 10, 12, 9
4-hydroxy Proline	0.61	132.10	68.11, 86.08	14, 12, 8
Leucine	1.67	132.10	69.2, 86	20, 10
Isoleucine	1.54	132.20	69.2, 86,1	20, 9
Asparagine	0.59	133.10	74, 87.13, 115.1	15, 10, 10
Aspartic acid	0.60	134.10	74, 88	14, 10, 8
Lysine	0.58	147.00	84, 115, 130.1	20, 12, 10
Glutamine	0.58	147.10	84.1, 130,1	16, 10
Glutamic acid	0.61	148.10	84, 102.1, 130.2	15, 12, 8
Methionine	1.00	150.20	56.1, 104.1, 133.2	15, 10, 9
Histidine	0.56	156.10	83.1, 93.1, 110.19	22, 20, 15
Phenylalanine	3.41	166.20	77, 91.2,103.1,120	30, 30, 25, 14
Arginine	0.57	175.20	60, 70, 116	15, 20, 15
Tyrosine	1.35	182.16	123.1, 136.1, 165.06	15, 15, 9
Tryptophan	4.27	205.10	91, 118.1, 188.16	35, 25, 10
Cystine	0.58	241.30	74, 120, 152	25, 20, 12

Table 1. Chromatographic and MRM method parameters for free amino acids using UPLC-ESI-MS/MS [1].

Phenolic compounds	Quantification > Confirmatory transition (m/z)	Cone (V)	Collision energies (V)	Mode
Pyrogallol	125.01 > 69.10, 79.04, 81.02	20	17, 17, 14	ESI (-)
Homogentisic acid	167.03 > 123.03, 122.08, 108.00	10	20, 20, 10	ESI (-)
Protocatechuic acid	153.06 > 108.00, 81.01, 91.01	10	20, 25, 20	ESI (-)
Gentisic acid	153.05 > 109.04, 108.03, 81.00	10	20, 20, 12	ESI (-)
Pyrocatechol	153.06 > 81.01, 108.00, 109.04	8	20, 25, 20	ESI (-)
Galantamine	288.10 > 198.00, 213.09, 230.95	20	32, 23, 17	ESI (+)

Phenolic compounds	Quantification > Confirmatory transition (m/z)	Cone (V)	Collision energies (V)	Mode
p-hydroxy benzoic acid	136.98 > 93.03, 65.10	10	25, 14	ESI (-)
3,4-dihydroxybenzaldehyde	137.00 > 91.93, 107.94, 136.00	8	21, 20, 18	ESI (-)
Catechin hydrate	288.88 > 109.15, 124.99, 245.26	30	25, 20, 15	ESI (-)
Vanillic acid	166.98 > 151.97, 108.03, 123.03	20	18, 12, 14	ESI (-)
Caffeic acid	179.10 > 135.14, 107.10, 133.9	32	23, 23, 24	ESI (-)
Syringic acid	197.20 > 123.00, 167.00, 182.00	15	22, 18, 14	ESI (-)
Vanillin	150.95 > 135.94, 91.90, 107.97	30	20, 20, 14	ESI (-)
p-coumaric acid	189.18 > 151.00, 203.00, 205.00	20	20, 20, 20	ESI (-)
Ferulic acid	163.01 > 119.04, 93.00, 117.01	5	27, 27, 15	ESI (-)
Epicatechin	193.03 > 134.06, 178.00, 149.02	20	16, 12,13	ESI (-)
Catechin gallate	441.00 > 168.98, 288.97	30	20, 20	ESI (-)
Rutin	609.00 > 254.99, 270.93, 299.90	17	55, 55, 40	ESI (-)
trans-2-hydroxy cinnamic acid	163.04 > 119.04, 117.01, 93.07	10	25, 22, 13	ESI (-)
Myricetin	316.90 > 107.07, 137.01, 150.97	30	30, 25, 25	ESI (-)
Resveratrol	227.01 > 143.01, 159.05, 185.03	30	25, 18, 18	ESI (-)
Trans-cinnamic acid	146.98 > 103.03, 62.18	30	10, 10	ESI (-)
Luteolin	284.91 > 107.01, 133.05, 151.02	20	30, 33, 30	ESI (-)
Quercetin	303.00 > 137.00, 153.00, 229.00	20	30, 32, 30	ESI (+)
Naringenin	270.98 > 107.00, 119.04, 150.97	20	25, 25, 20	ESI (-)
Genistein	271.00 > 153.00, 215.00, 243.00	20	27, 25, 24	ESI (+)
Apigenin	269.10 > 107.00, 117.00, 149.00	20	30, 30, 25	ESI (-)
Kaempferol	284.90 > 158.97, 117.10, 227.14	10	34, 40, 30	ESI (-)
Hesperetin	301.02 > 108.01, 136.00, 163.99	20	36, 30, 24	ESI (-)
Chrysin	252.99 > 63.05, 107.05, 142.99	20	30, 25, 25	ESI (-)

Table 2. Chromatographic and MRM method parameters for the analysis of phenolic compounds using UPLC-ESI-MS/MS [2].

Total ion chromatograms (TIC) of each analyte are displayed in **Figures 1** and **2** for amino acids and phenolic compounds, respectively.

2.2. Extraction procedure for amino acid analysis

To prepare 10% (m/v) water honey solutions, 20% methanol solution (v/v) (20 mL), initially acidified with 0.1% formic acid (v/v), is added to 2.0 g honey samples. The resulting mixtures are placed in an ultrasonic bath at 36°C for 10 min to completely mix the

Column	**C_{18} column (1.7 μm 2.1 × 100 mm)**			
Mobile Phase A	0.1% aqueous formic acid			
Mobile phase B	methanol/water (50:50, v/v) containing 0.1% formic acid			
Column oven temp.	40°C			
Injection volume	1 μL			
Gradient	Time (min)	Flow (mL/min)	Mobil phase A (%)	Mobil phase B (%)
	0.00	0.400	99.00	01.00
	2.00	0.400	99.00	01.00
	8.00	0.400	30.00	70.00
	9.00	0.400	99.00	01.00
	10.00	0.400	99.00	01.00

Table 3. Chromatographic conditions for free amino acid analysis [1, 12, 16].

Column	**C_{18} column (1.7 μm 2.1 × 100 mm)**			
Mobile phase A	0.5% (v/v) acetic acid in ultrapure water			
Mobile phase B	0.5% (v/v) acetic acid in acetonitrile			
Column oven temp.	40°C			
Injection volume	2 μL			
Gradient	Time (min)	Flow (mL/min)	Mobile phase A (%)	Mobile phase B (%)
	0.00	0.650	100.00	00.00
	1.00	0.650	99.00	01.00
	10.00	0.650	70.00	30.00
	12.00	0.650	5.00	95.00
	13.00	0.650	99.00	01.00
	14.00	0.650	100.00	00.00

Table 4. Chromatographic conditions for phenolic compound analysis [2, 11, 12].

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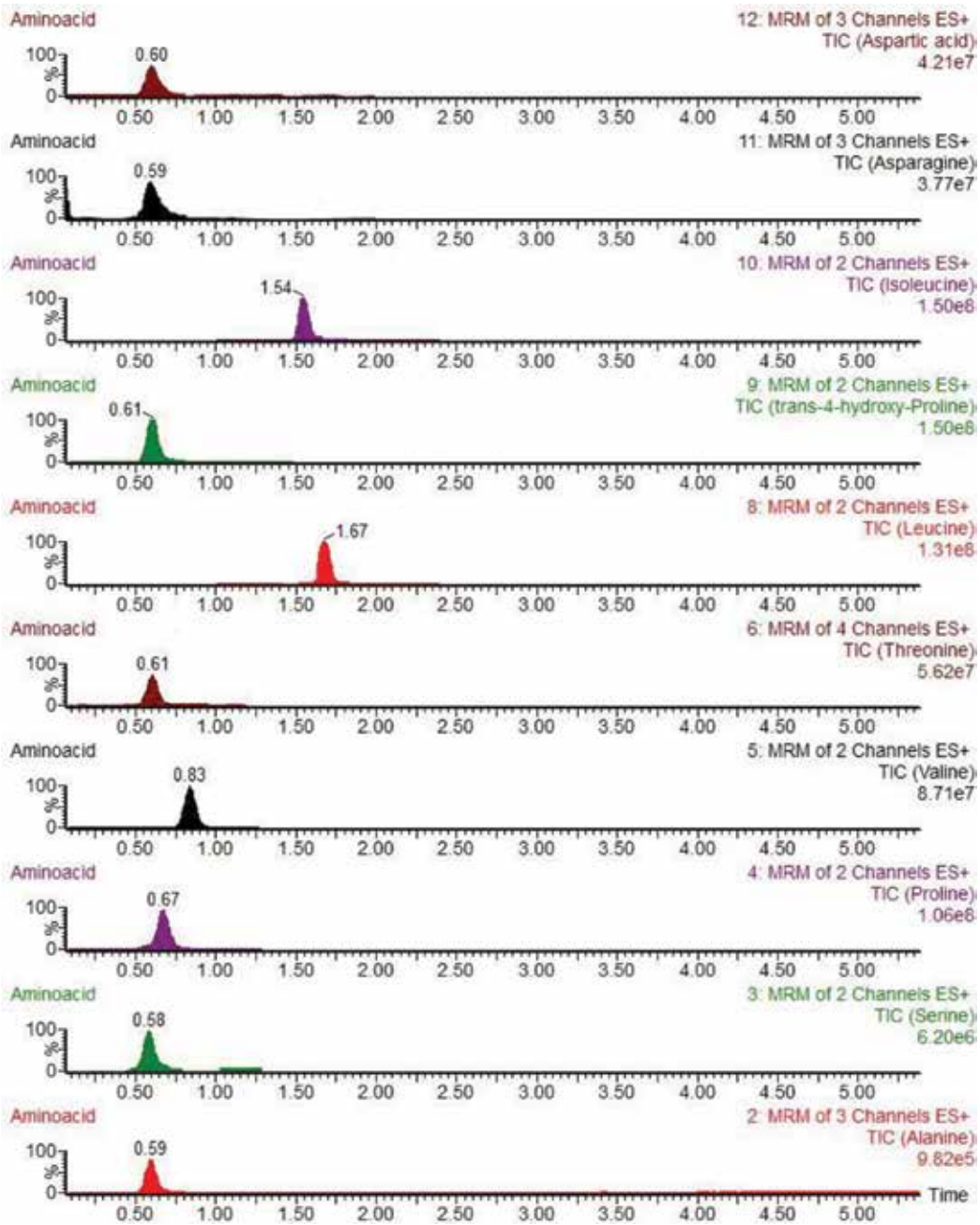
extracts of analysed honey samples and subsequently centrifuged at 4000 rpm and 4°C, then the supernatant is filtered through 0.20-μm-pore diameter polytetrafluoroethylene (PTFE) membranes to remove any solid particles, and added to vials and injected into UPLC-ESI-MS/MS [1].



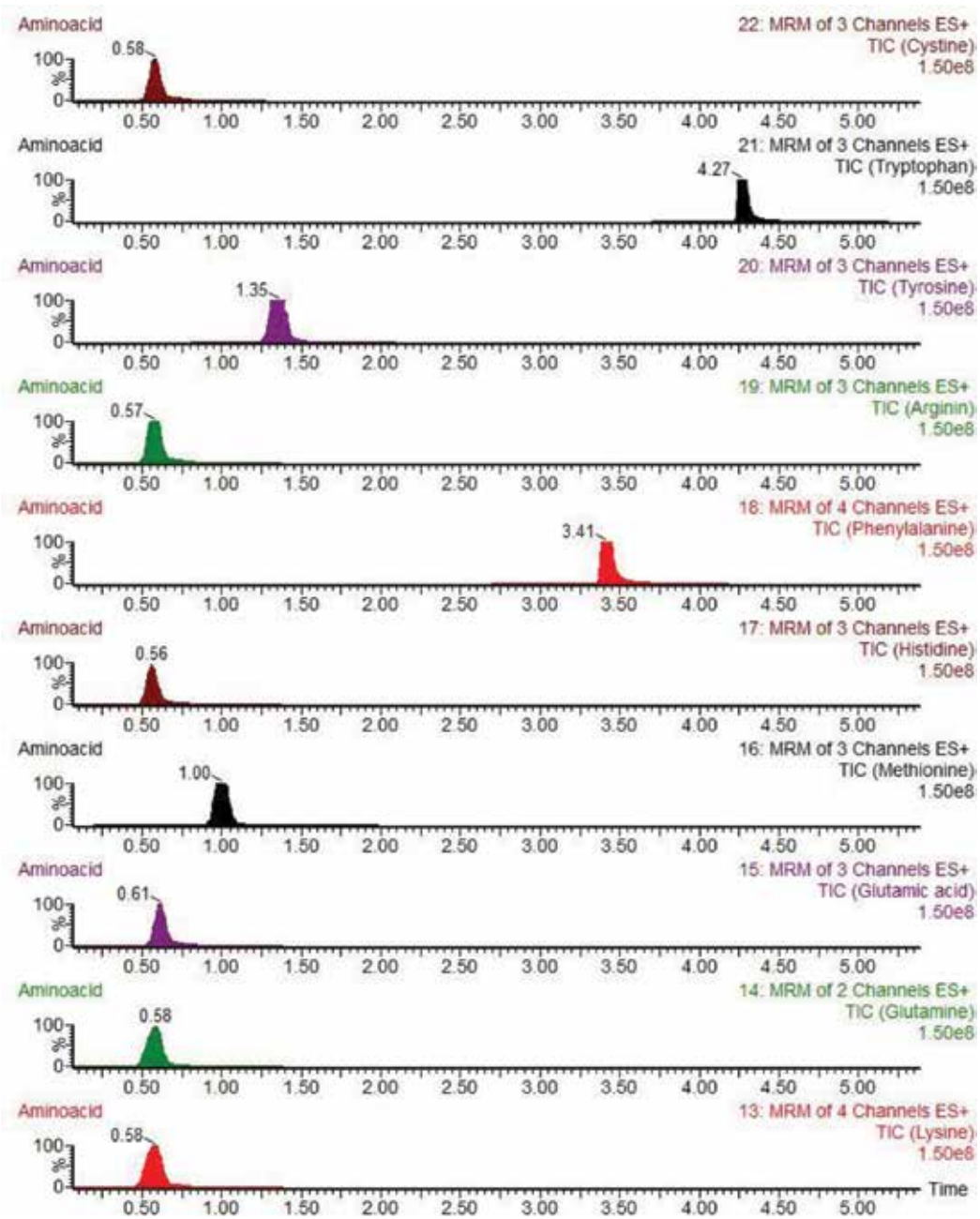


Figure 1. Total ion chromatograms (TIC) of free amino acids using UPLC-ESI-MS/MS.

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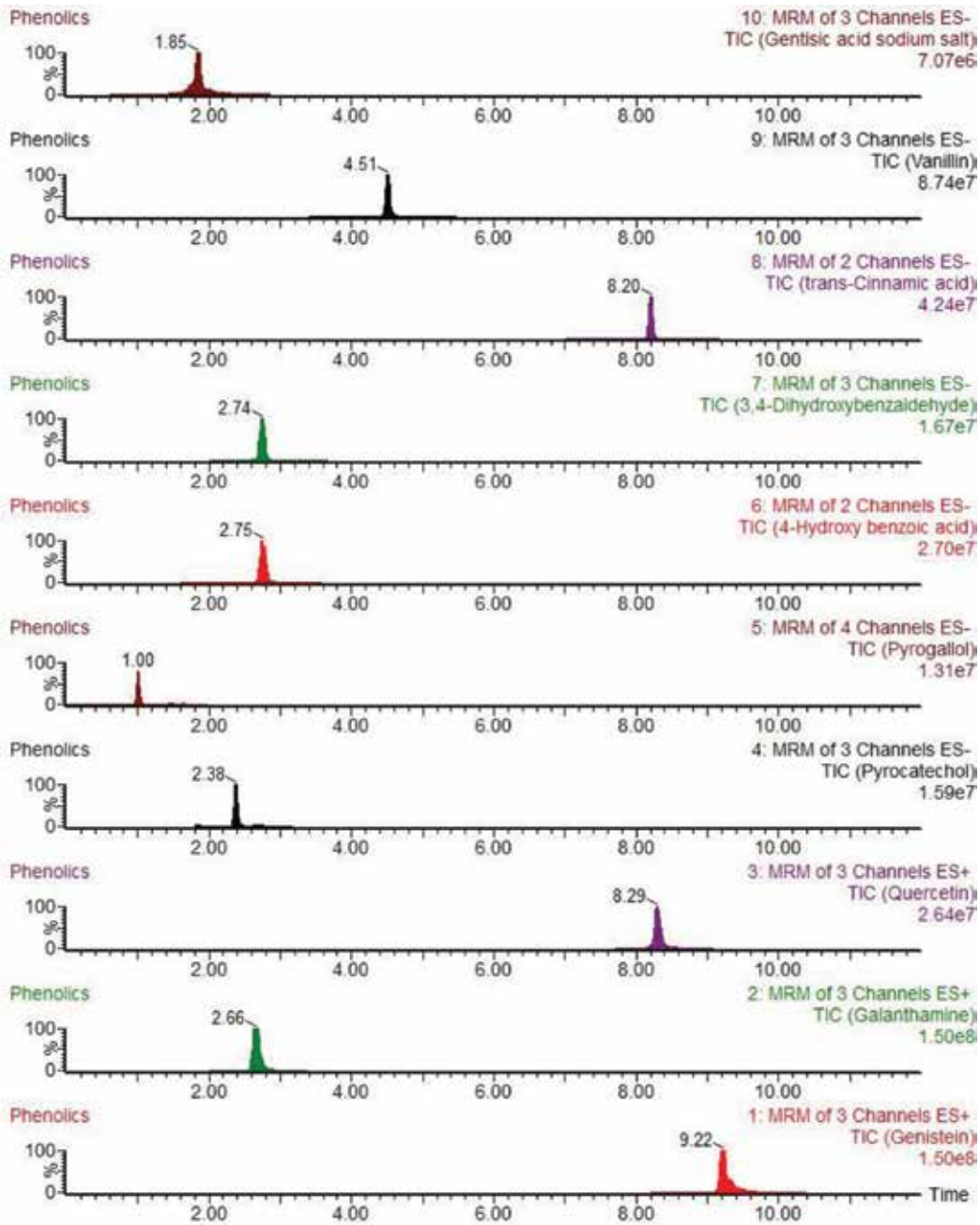

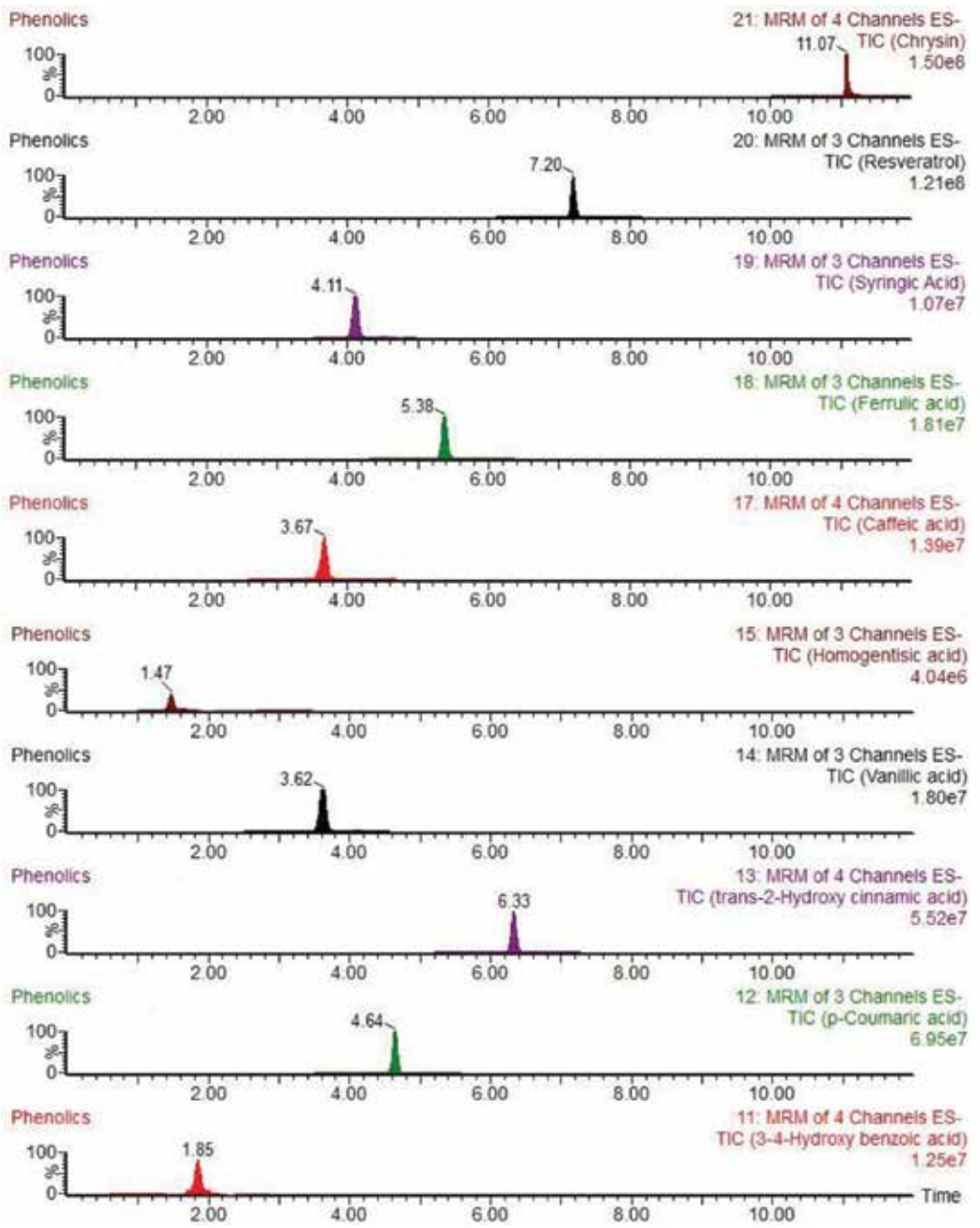

Figure 2. Total ion chromatograms (TIC) of phenolic compounds using UPLC-ESI-MS/MS.

2.3. Extraction procedure for phenolic compound analysis

Honey sample (10 g) is dissolved in ultrapure water (50 mL) and mixed 5 min via vortex. Then ethyl acetate (50 mL) is added into solution flask and the flask is placed on a shaker for 30 min.

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After then, the flask settles for the phase separation for 180 min. The water phase is extracted two more times with ethyl acetate, and the combined ethyl acetate extract is evaporated under vacuum at 36°C. The residue is redissolved in methanol (5 mL) and filtered from polytetrafluoroethylene (PTFE) membrane 0.20 μm and added to vials, and 2 μL of the solution is injected into UPLC-ESI-MS/MS [2].

3. Discussion

In the analysis of amino acids and phenolic compounds, an extraction procedure is important. Extraction of amino acids and phenolic compounds depends on their chemical properties such as natural matrix and molecular structure together with their polarity, concentration, aromatic chain number and variation in their hydroxyl groups, etc. Protein, carbohydrate and other complex structures are hindered to extract several phenolic compounds. Differences in the chemical structure of phenolics in the sample are related to concentration of the functional groups, simple and complex polyphenolic structures and phenolic acid and flavonoid in different proportions. In the literature, more than one methods and techniques are needed to be used for the extraction.

The extraction step of amino acids and phenolic compounds is the very critical step after sample preparation. Organic and inorganic solvents are commonly used in the extraction. The efficiency of the extraction is affected from including extraction temperature, time, solvent-sample ratio and solvent types.

Furthermore, treatment time and temperature together with a selection of solvent ratio is very crucial for optimum recovery of amino acids and phenolic compounds. Generally, increasing time and temperature is preferable for the solubility of analytes; however, undesirable enzymatic oxidation arising from high temperature and extended extraction time may cause degradation of amino acids and phenolic compounds. The solvent–sample ratio and repetition number of extraction affect the recovery of phenolic compounds for each sample [17].

Sample matrix and particle size are highly affected the extraction of amino acids and phenolics. Issue of diffusion is related to particle size. Diffusion becomes easier as particle size gets smaller and efficiency of extraction gets higher. However, this increasement continues to some level and after that point it stops or decreases. That situation shows up with the reduction of the mass transfer rate caused by small particles. More solvent is needed in this stage.

Phenolic substance can bound to organic bodies such as carbohydrate and protein in the sample materials. And thus, bounded phenolics can be liberated by hydrolysis with the addition of enzyme.

According to literature survey, there is a lack of knowledge about the profiles of amino acids and phenolic contents in honey to evaluate the quality of the product.

Several studies have been revealed that honey serves as a source of natural antioxidants with the anti-microbial, anti-inflammatory, anti-mutagenic, anti-tumour and anti-oxidative activity, which are effective in reducing the risk of heart disease, immune-system decline, different

inflammatory processes, etc. [2]. Honey species also possess antibacterial activities and are scavengers of active oxygen radicals [15]. Among the components present in honey which are responsible for its anti-oxidative effect are phenolic compounds (flavonols, flavones, flavanones, benzoic and cinnamic acids) [17].

Thereby, as honey is a very complex product. Depending on the nectar-providing plant species, bee species, geographical area, season and a method of storage demand a comprehensive analysis of constituents, such as volatile compounds, phenolic acids, flavonoids, carbohydrates and amino acids, for its characterization [2, 18].

According to literature survey, arginine, tryptophan, phenylalanine, tyrosine and lysine are found in considerable amounts in honey. And also in various studies, they are qualified as a characteristic of some floral types of honey [18, 19].

Phenylalanine, proline, tyrosine, isoleucine, and leucine are revealed as the main amino acids [1]. The studies indicate, on the basis of honey activity, a better differentiation, considering free amino acid contents instead of physicochemical honey characteristics [19]. Moreover, amino acid composition may also be a suitable method to determine honey botanical origin [1, 20].

Around 200 substances have been reported in this complex natural liquid but the composition especially its secondary metabolites and quality of honey may be influenced by some external factors such as environmental and seasonal factors, processing, handling and storage [5, 6].

The determination and evaluation of phenolic constituents in honey appeal high attention by consumers and researchers owing to a health-promoting feature that is accompanied by bioactivity [21].

Botanical origin of honey is classified according to phenolic ingredients [21, 22] and this consequently implies that as honeybees collect nectar from plants which contain bioactive components. These phytochemical ingredients can be transferred to honey by honeybees [23, 24]. Numerous flavonoids (such as apigenin, kaempferol, quercetin, chrysin and luteolin) and phenolic acids (caffeic, gallic, cinnamic, protocatechuic, p-coumaric and chlorogenic acids) are identified in various honey samples [2].

4. Conclusion

The studies displayed that the UPLC-ESI-MS/MS instrument demonstrates to be reliable for the unambiguous detection of a large number of compounds, by enabling the determination of amino acids and phenolic profiles of honey.

Currently, most studies that provide information on honey are directly related to the quality parameters, there are not many studies that analyse chemical compounds present in the honey. Therefore, this research is needed to found a control system that evaluates maintenance of the characteristics and levels of those compounds provided by honey to human nutrition and health. Thereby increasing the levels of security in quality, generating reliability for consumers and ensuring honey consumption devoid of toxic compounds for human health.

The effective technique for identifying the natural nature of a honey is the amino acid analysis. Amino acid analysis of honey is a promising technique in the evaluation of the botanical origin. Thus, honey is described with a good level of complacency.

The rapid, accurate determination and identification phenolic compounds in honey are provided by an improved and easy analytical. Both of the methods proved to be effective for determining honey quality.

Nevertheless, apart from the delicious sweet taste of honey, being the crucial source of free amino acids and phenolic compounds, honey can also be consumed as supplementary materials for food products and applied in nutrients, cosmetics, and pharmaceutical industries.

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Chapter 5

Fluorescence: A Novel Method for Determining Manuka Honey Floral Purity

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Additional information is available at the end of the chapter

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Abstract

Manuka honey, harvested from *Leptospermum scoparium*, is New Zealand's most recognised honey type and commands a premium due to health-related benefits. However, the plant's distribution, relative to other species flowering simultaneously, allows honeybees to incorporate alternative nectars into the honey. Melissopalynological analysis in New Zealand is often unrepresentative due to the presence of many pollen-bearing sources; consequently, alternative means of categorising manuka honey were examined. RP-HPLC revealed that manuka honey contains distinct compounds, of which were relatively enriched and not present in the other New Zealand monofloral honeys. These main candidate compounds were isolated and have been described by mass spectrometry and nuclear magnetic resonance, synthesised to confirm structure, and as standards. These compounds, Leptosperin and Lepteridine, are a methyl syringate glycoside and pteridine derivative, respectively. Examination of these compounds revealed unique fluorescence signatures, this fluorescence could be detected in manuka honey samples the signal used to confirm that a honey was solely or predominantly consisted of *L. scoparium* nectar. Commercial manuka honeys were assessed by traditional analytical techniques, and comparisons were made with fluorescence signature; the fluorescence technique determined the authenticity of the honeys accurately.

Keywords: Honey, Floral, Fluorescence, Manuka, *Leptospermum scoparium*, Authenticity, purity

1. Introduction

New Zealand manuka honey is harvested from *Leptospermum scoparium* (Myrtaceae) throughout the country. Internationally, this honey has received considerable attention and value due to its unique health-related benefits. Major destination markets include Hong Kong and China, Japan, the European Union, United Kingdom, the United States and Australia. Over 80% of the total honey exported from New Zealand is now pre-packaged, hive numbers in the country have almost doubled in the last 10 years [1] and the value of the manuka honey industry is now estimated in the vicinity of NZ$150 million.

Codex Alimentarius [2] defines that a honey must be derived wholly or predominantly from a particular floral source and display the corresponding organoleptic, physico-chemical and microscopic properties for a floral attribution to be made. Within New Zealand, a number of surplus nectar-producing common plant species exist with similar distributions and flowering times as *L. scoparium*. Consequently, manuka honey may contain different levels of dilution by other floral types, as honey produced in a natural environment containing a range of plant species is unlikely to be monofloral because of bee behaviour in the forage field [3].

Historically, New Zealand honeys have been classified by physico-chemical analysis and melissopalynology. Melissopalynology is a common technique internationally for describing honeys; however, in New Zealand *Kunzea ericoides* often flowers simultaneously with *L. scoparium*, and the pollen grains of these species are virtually indistinguishable in a honey medium [4]. To overcome this, a classification structure was built upon the unique non-peroxide antibacterial activity that manuka honey exhibits, yet this system did not take into the account of honey's floral composition.

Honey is a complex supersaturated sugar solution containing approximately 80% sugars and a unique combination of other compounds suspended in water. The sugar proportion is principally the monosaccharide fructose and glucose, and the non-sugar proportion includes a range of bee- and plant-derived compounds such as organic acids, proteins, amino acids, phenolic acids, flavonoids, pollen and waxes [5]. This chemical composition varies between honey types, geographical origin and climate may additionally alter the constituents [5], and furthermore honey processing techniques and age may also be influential [6].

Manuka honey contains a diverse array of compounds that range from unique carbohydrate metabolites to phenolics, flavonoids and volatiles. Many of these have received attention [7–11], and clearly, this honey carries a number of distinct compounds that may be diagnostic for classification. For example, 2-methoxyacetophenone (**Figure 1**) [7, 11] and 2-methoxybenzoic acid [10, 11] have been proposed as floral markers for manuka honey.

In addition, dihydroxyacetone (DHA) and methylglyoxal (MGO) (**Figure 1**) are solely derived from *L. scoparium* nectar in New Zealand honeys [12, 13]. Dihydroxyacetone is present in *L. scoparium* nectar, converting non-enzymatically and irreversibly to MGO in the acidic environment of a ripened honey solution [13]. This conversion is non-stoichiometric [14, 15] due to the presence of side reaction pathways in the honey. However, the concentrations of these compounds are not stable throughout a manuka honey's shelf-life and therefore neither are suitable as reliable chemical markers [6].

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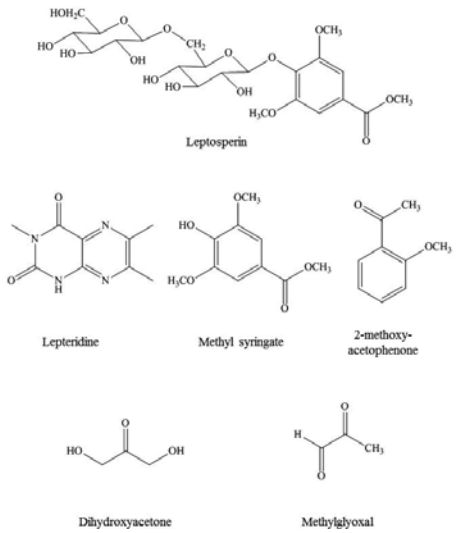

Figure 1. Chemical structure of Leptosperin, Lepteridine, 2-methoxyacetophenone, methyl syringate, dihydroxyacetone and methylglyoxal.

Internationally, classifying honeys by chemical signature or key components has received increasing attention over the last 20 years. European honeys have been thoroughly investigated [16] confirming earlier work on, for example, rosemary [17] and heather [18, 19] honeys.

Further investigation on the phenolic and flavonoid profile of manuka honey has revealed two unique compounds. First, a nectar-derived glycoside of methyl syringate has been described [20, 21]. Whilst this compound is present in the wider *Leptospermum* genus throughout Australasia, it is restricted to *L. scoparium* in New Zealand and therefore is potentially a suitable floral marker. Consequently, methyl syringate 4-O-β-D-gentiobiose in manuka honey, named Leptosperin[1] (**Figure 1**), has been analysed by high-performance liquid chromatography (HPLC), mass spectrometry, immunochemistry and immunochromatography [21–24].

More recently, analysis showed the presence of another unique compound in *L. scoparium* nectar and honey. In this case, the compound was described as a pteridine derivative 3,6,7-trimethyllumazine, and named Lepteridine (**Figure 1**) [25]. This compound has also been quantified by HPLC in manuka honey [26]. Both Leptosperin and Lepteridine have been reported to be chemically stable over prolonged storage in honey [21, 22, 26].

Methyl syringate (**Figure 1**) has also been shown to be present at elevated concentrations in manuka honey. However, previous studies indicate that this compound does not correlate with non-peroxide activity [20, 21]. Additionally, methyl syringate concentration has also been reported as elevated in kanuka honeys (*K. ericoides*) and is higher than that reported in manuka honeys [7, 10]. Accordingly, methyl syringate may not be a suitable chemical marker for manuka honey.

[1]Leptosperin was initially named 'leptosin' [20] but was later renamed to avoid confusion with the marine fungus-derived leptosins [21].

Beyond traditional analytical techniques, fluorescence spectroscopy has demonstrated use in analysing a range of food products including honeys [27–30]. Fluorometric methods are reported to be up to 1000 times more sensitive than absorption-based techniques [31]. Fluorescence spectroscopy provides improved specificity by examining distinct excitation and emission wavelengths and is a rapid, cost-effective and efficient non-destructive method [32, 33].

Fluorescence in honeys has been attributed to phenolic and polyphenolic compounds [27–30], amino acids [28–30] and Maillard reaction products [28, 29]. As phenolic and polyphenolic compounds have been described as reliable indicators of botanical and geographical origin of honeys [10, 16, 34, 35]; the fluorescence properties of these intrinsic and unique fluorophores may inform identification of floral source reliably.

Recent examination of the fluorescence profiles of the main New Zealand honey types demonstrated that manuka honey exhibited unique fluorescence characteristics that distinguish it from the other honey types [36]. Manuka honey contained two unique fluorescence signatures, $_{ex}270–_{em}365$ nm and $_{ex}330–_{em}470$ nm, named MM1 and MM2, respectively [36]. Dilution of manuka honey with other New Zealand honey types, which did not fluoresce at the diagnostic wavelengths, resulted in a reduction of the fluorescence signal in the manuka honey that was proportional to the dilution.

Further work confirmed that Leptosperin was responsible for the MM1 fluorescence signature ($_{ex}270–_{em}365$ nm) [22] and Lepteridine was the principal compound associated with MM2 fluorescence ($_{ex}330–_{em}470$ nm) in manuka honey [26]. For these compounds, standards were synthesised for Leptosperin [37] and Lepteridine [25], and seeding of honeys experimentally confirmed that these compounds are the primary fluorophores.

Consequently, these findings demonstrate manuka honey contains unique fluorophores that may be quantified to establish floral authenticity. As this technique is fluorescence-based, it provides the opportunity for rapid screening of honey samples to confirm honey labelling is appropriate and complies with the wholly or predominantly ruling in Codex Alimentarius [2]. In this chapter, the fluorescence technique is applied to sets of field-collected manuka honeys and a set of manuka honeys purchased commercially in 2016. Other compounds of interest in manuka honey, such as 2-methoxyacetphenone, methyl syringate, MGO and DHA, are additionally quantified.

2. Fluorescence markers in manuka honey

The fluorescence markers in manuka honey were assessed in a number of honey collections, first, field honeys harvested from *L. scoparium* hive sites in New Zealand, and second, a commercial set purchased from retail distributors in Singapore. The honeys in the purchased set were labelled as manuka honey and therefore should be wholly or predominantly sourced from *L. scoparium*.

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2.1. Leptosperin

Leptosperin has been shown to be uniquely derived from the *Leptospermum* genus in New Zealand and is present in manuka nectar and honey. This compound is readily quantified by liquid chromatography and mass spectrometry techniques and has been recently shown to be primarily responsible for the fluorescence exhibited by manuka honey at MM1 wavelengths [22]. As Leptosperin has been demonstrated to be chemically stable during extended storage experiments [22], this compound is an ideal candidate as a chemical and fluorescence marker for manuka honey.

Leptosperin is present in manuka honey at a concentration up to approximately 1700 mg/kg, with a minimum reported concentration of 93 [22] to 126 mg/kg [20], and therefore, it is probable that manuka honey can be expected to carry a minimum of 100 mg/kg. Fluorescence of Leptosperin is readily detected in manuka honey using the reported technique with lower detection limit of 10 ppm.

The field collected manuka honeys (n = 28) and the commercial honeys (n = 17) exhibited fluorescence that strongly correlated (R^2 = 0.9530) with the quantified concentration of Leptosperin (**Figure 2A**), confirming the previous research of this compound. The concentration of Leptosperin in the commercial samples fell in the lower half of the range recorded for the field samples. The mean concentration of Leptosperin was 423 and 192 mg/kg in the field and commercial samples, respectively ($p < 0.0001$). This is consistent with the previously reported comparison of field and commercial manuka samples [22]. However, two of the commercial samples contained less than 100 mg/kg Leptosperin which is considered to be the lower than acceptable minimum concentration.

Leptosperin and MM1 analysis of an additional field honey collection (n = 71) throughout New Zealand (**Figure 2B**) demonstrated that each region contained honeys that were distrib-

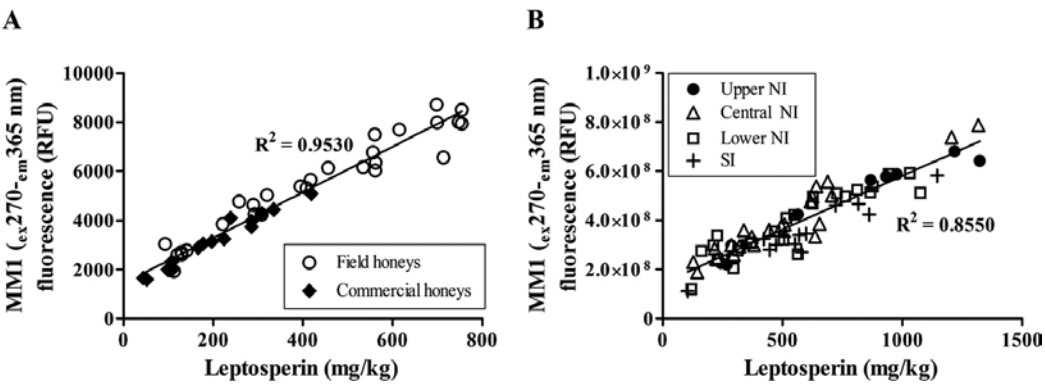

Figure 2. (A) Correlation between Leptosperin concentration and MM1 fluorescence in field and commercial manuka honeys. (B) Regional distributions of Leptosperin concentration and MM1 fluorescence, all data correlation shown.

uted throughout the range of recorded concentrations and signal. These observations reinforce the earlier findings that Leptosperin is a reliable fluorescence marker in manuka honey and can be used to categorise the national crop.

2.2. Lepteridine

Lepteridine is also uniquely derived from the *Leptospermum* nectar in New Zealand and appears to be present in all manuka honeys. This compound has been quantified by liquid chromatography, and is principally responsible for the fluorescence exhibited by manuka honey at MM2 wavelengths [26]. Lepteridine has also been shown to be chemically stable in elevated storage temperature [26] and has been proposed as an additional chemical and fluorescence marker for manuka honey.

The concentration of Lepteridine in field collected manuka honey is reported to be in the range between 5 and 50 mg/kg [26] and has not been examined in commercial samples previously. Concentrations as low as 1 ppm were detected by using the reported fluorescence method.

Again the field collected manuka honeys (n = 27) and the commercial samples (n = 17) displayed similar characteristics. The concentration of Lepteridine was correlated linearly (R^2 = 0.9433) with the fluorescence signal at MM2 (**Figure 3**). Nonetheless, the commercial samples contained significantly lower concentrations of Lepteridine than the field samples, being a mean value of 28 and 6 mg/kg, respectively ($p < 0.0001$) which is a fourfold difference. Furthermore, seven of the commercial samples contained less than the reported lower concentration of 5 mg/kg; four of which contained 4 mg/kg Lepteridine.

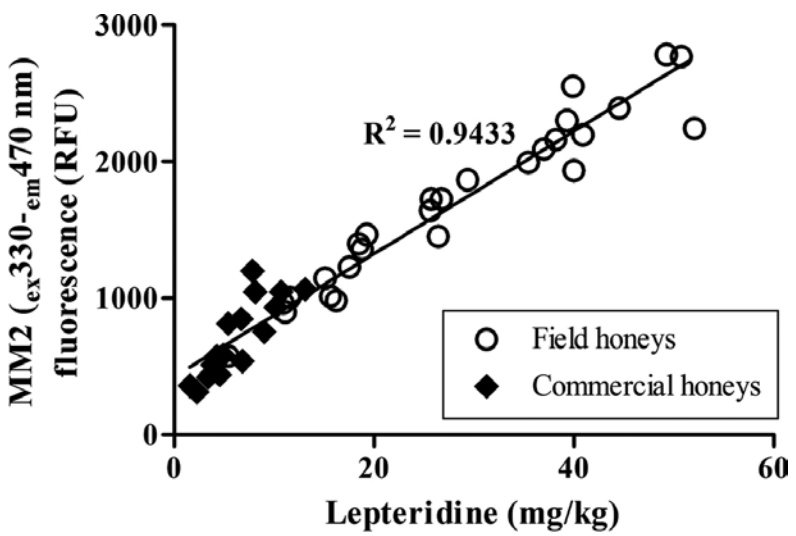

Figure 3. Correlation between Lepteridine concentration and MM2 fluorescence in field and commercial manuka honeys.

2.3. Correlation of MM1 and MM2 fluorescence signal

The fluorescence generated by Leptosperin and Lepteridine at MM1 and MM2 display a relatively strong linear correlation in manuka honeys (R^2 = 0.8620). This indicates a degree of colinearity between these compounds. Whilst this may limit the use of both compounds in model development using data from more traditional techniques such as liquid chromatography or mass spectrometry, the use of two independent wavelength pairs in fluoro-spectroscopy is expected to considerably strengthen this technique.

The fluorescence signal at both marker wavelengths in manuka field honeys (n = 27) is illustrated along with the commercial samples (n = 17) (**Figure 4**). The commercial sample mean fluorescence was significantly lower than the field samples for both MM1 ($p < 0.0001$) and MM2 ($p < 0.001$), and this reflects the concentration of Leptosperin and Lepteridine that are present in these commercial honeys.

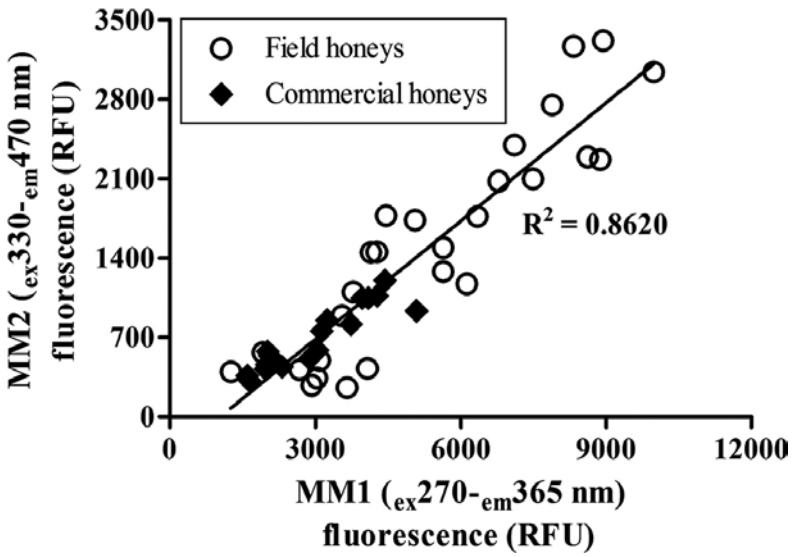

Figure 4. Correlation between MM1 and MM2 fluorescence signal in field and commercial manuka honeys.

3. Examination of non-fluorescent markers in commercial honeys

3.1. 2-Methoxyacetophenone and methyl syringate

2-Methoxyacetophenone has been described as a marker compound for manuka honey previously using HS-SPME-GC/MS [7, 11]. For the first time, this chapter reports the analysis of this compound in honey solutions using HPLC-DAD. The concentration of Leptosperin correlated strongly (R^2 = 0.8722) with 2-methoxyacetophenone concentration (**Figure 5A**) rein-

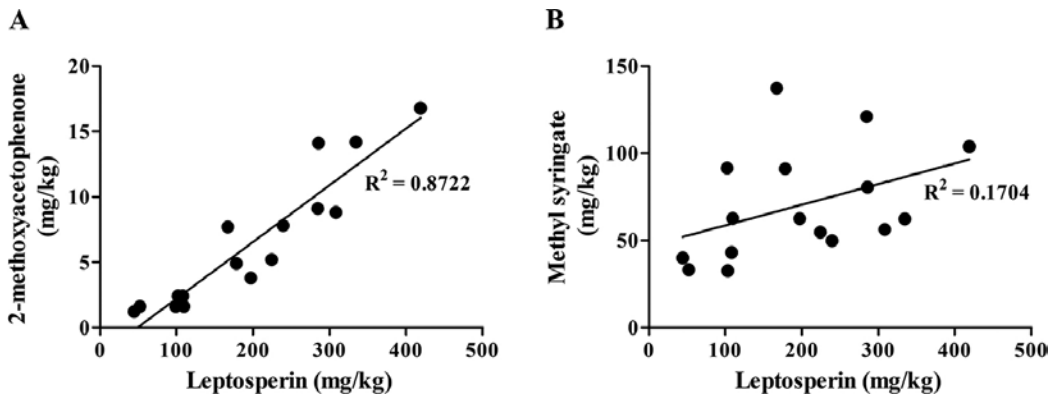

Figure 5. Correlation between Leptosperin and (A) 2-methoxyacetophenone and (B) methyl syringate in commercial manuka honey samples.

forcing the previous publications regarding the use of this compound as a potential marker for manuka honey.

Methyl syringate concentration is elevated in manuka and kanuka honeys in New Zealand [7, 10]. The linear correlation (**Figure 5B**) of Leptosperin and methyl syringate concentrations is poor (R^2 = 0.1704). This may be a reflection of kanuka content in manuka honeys; the kanuka honey is expected to contribute additional elevated levels of methyl syringate. Therefore, methyl syringate is not a reliable marker for manuka honey.

3.2. Dihydroxyacetone and methylglyoxal

Dihydroxyacetone is the precursor compound for MGO in ripening and maturing manuka honey. Dihydroxyacetone concentration in manuka honey can vary for three reasons: first, the DHA concentration in nectar harvested from the varieties of *L. scoparium* is significantly different, and this may vary as much as twofold [38], second, floral dilution will reduce the amount of DHA being incorporated into a honey during ripening and third, this precursor undergoes chemical reactions in the maturing honey solution [6, 15].

Conversely, MGO is absent in nectar. The MGO concentration increases rapidly during a manuka honey's first couple of years as the chemical conversion of DHA to MGO proceeds. However, the rate of conversion declines as the DHA pool is exhausted. Furthermore, in honeys that are reaching the end of their five-year shelf-life, it has been demonstrated that the MGO concentration begins to decline as insufficient DHA remains to sustain the MGO concentration [6].

These changes in DHA and MGO concentration are best demonstrated in elevated temperature storage experiments which promote the chemical reactions. Thirteen honeys stored for a little over 3 months at 37°C demonstrate these effects (**Figure 6A**). In this time, DHA decreased by about 18% and MGO increased by 46%, and these concentration shifts continue as the honey matures. Conversely, decreased storage temperature will significantly reduce

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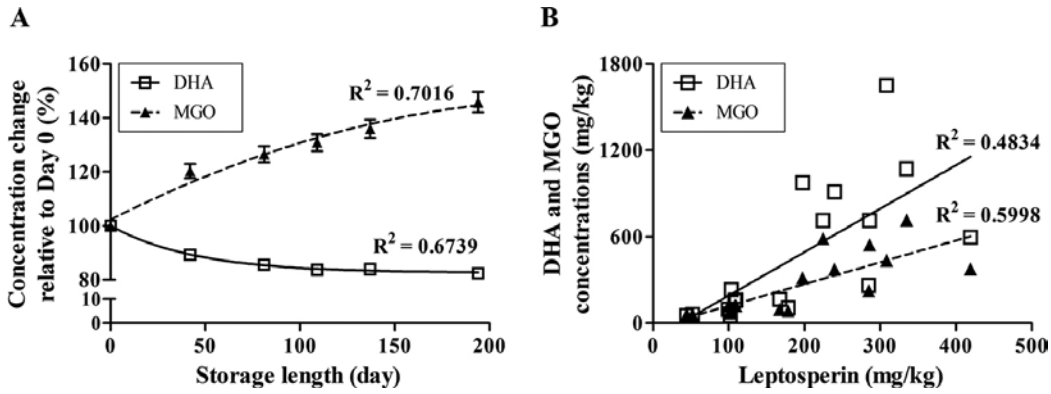

Figure 6. (A) The relative concentrations of DHA and MGO expressed as a percentage of the initial concentration in 10 manuka honeys stored at 37Â°C and (B) the relationship of DHA and MGO to the fluorescent marker Leptosperin in commercial manuka honeys.

the rate of chemical reactions in the honey; therefore, the concentration of DHA and MGO in a manuka honey can be considerably influenced by processing and storage conditions.

Leptosperin and Lepteridine have also been quantified in honeys stored at elevated temperatures for over 400 days and neither compound demonstrated any significant deviation from the initial concentration [22, 26].

For manuka honey harvested from hives in a particular well-defined region where the DHA concentrations in the nectar is relatively constant, there is a relatively strong correlation between MM1 fluorescence and DHA concentration [39]. This is because the honeys are of a uniform age and therefore exclude ageing differences, the DHA potential of the nectar is relatively similar as the harvested *L. scoparium* population is very discrete and genetically linked, and any reduction of DHA in the honey can be attributed to floral dilution from other nectar sources. Consequently, the floral dilution alone acts upon the fluorophores such as Leptosperin, resulting in a relatively stronger correlation between DHA and Leptosperin in the honey.

However, when commercial manuka honey samples are considered the effect of both different initial DHA concentrations in nectar, and ageing-driven chemical reactions, which may occur at different rates due to temperature influences, are very difficult to separate from floral dilution. The concentrations of Leptosperin, DHA and MGO (**Figure 6B**) for commercial manuka honeys demonstrate the relatively poor correlation that exists in commercial honeys of different provincial provenance, unknown age and storage conditions.

The concentrations of DHA and MGO relative to Leptosperin (**Figure 7**) in the 71 field honeys shown in **Figure 2B** reinforce the insignificant relationship between these compounds and a consistent floral marker. The regional data groups are scattered and there is no significant linear correlation as it is likely that the genetic linkage within these large regional areas is insufficient to overcome variability between DHA potential in *L. scoparium* nectar. Similarly,

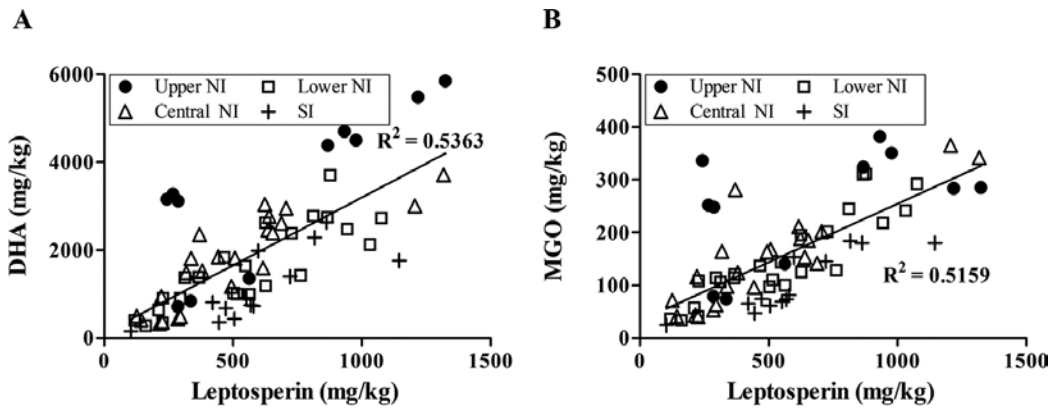

Figure 7. The relationship of (A) DHA and (B) MGO to the fluorescent marker Leptosperin in field honeys throughout New Zealand.

MGO displays a poor correlation with Leptosperin, where differences in ageing profile as well as initial DHA potential are magnified.

Therefore, despite both DHA and MGO being unique to manuka honey in New Zealand, neither of these compounds are reliable predictors of floral authenticity in commercial manuka honey samples. Both DHA and MGO should be present in genuine manuka honey; however, the concentration of these compounds does not correlate with stable nectar-derived chemical markers.

3.3. Commercial manuka honey samples summary

The concentrations of Leptosperin, Lepteridine, 2-methoxyacetophenone, methyl syringate, DHA and MGO, along with MM1 and MM2 fluorescence and Unique Manuka Factor (UMF) are presented in **Table 1**. UMF is the non-peroxide antibacterial grading system devised 25 years ago that measured bioactivity [40] and did not take into the account of floral authenticity.

The significant manuka chemical markers vary in concentration up to more than 1 order of magnitude in the examined honey samples. The harvested *L. scoparium* variety and floral dilution by other plant species contribute to this range. However, three of the analysed honeys, Samples #1, #2 and #3, demonstrated chemically low or marginal results for Leptosperin and Lepteridine, and likewise had particularly low concentrations of DHA and MGO.

Box- and whisker-plots and histograms (**Figure 8**) demonstrate the statistical distribution of Leptosperin and Lepteridine in the commercial honey set. Both datasets are positively skewed, with mean values greater than the median, and this confirms that more manuka honey in this set has greater concentrations than the proposed lower limits for Leptosperin and Lepteridine.

Sample	Concentration (mg/kg)						Fluorescence (RFU)		UMF
	Leptosperin	Lepteridine	2-MAP	MSYR	DHA	MGO	MM1	MM2	
1	44	2.28	1.18	40	52	59	1649	314	4.1
2	52	1.57	1.59	33	57	59	1594	359	4.1
3	102	3.37	2.44	91	50	69	1973	422	4.5
4	99	4.28	1.61	205	94	117	1998	458	6.1
5	103	6.82	1.87	33	231	103	2058	539	5.7
6	108	4.54	2.37	43	157	124	2319	438	6.4
7	110	4.25	1.56	63	160	121	2014	574	6.3
8	167	3.82	7.72	137	165	94	2885	511	5.4
9	178	4.89	4.88	91	104	84	3052	584	5
10	197	9.00	3.82	62	976	313	3135	754	11.1
11	224	6.73	5.20	55	712	585	3245	849	16.2
12	239	8.13	7.82	50	911	372	4097	1047	12.3
13	285	5.40	9.09	121	262	224	3735	814	9.1
14	286	10.73	14.14	81	711	542	3970	1046	15.5
15	308	13.13	8.75	56	1650	435	4277	1066	13.6
16	335	7.85	14.15	62	1070	715	4438	1199	18.3
17	419	10.15	16.83	104	594	375	5087	933	12.4

Note: 2-MAP, 2-methoxyacetophenone; MSYR, methyl syringate; DHA, dihydroxyacetone; MGO, methylglyoxal.

Table 1. Chemical composition, fluorescence, and non-peroxide antibacterial activity (UMF) of 17 commercial manuka honey samples.

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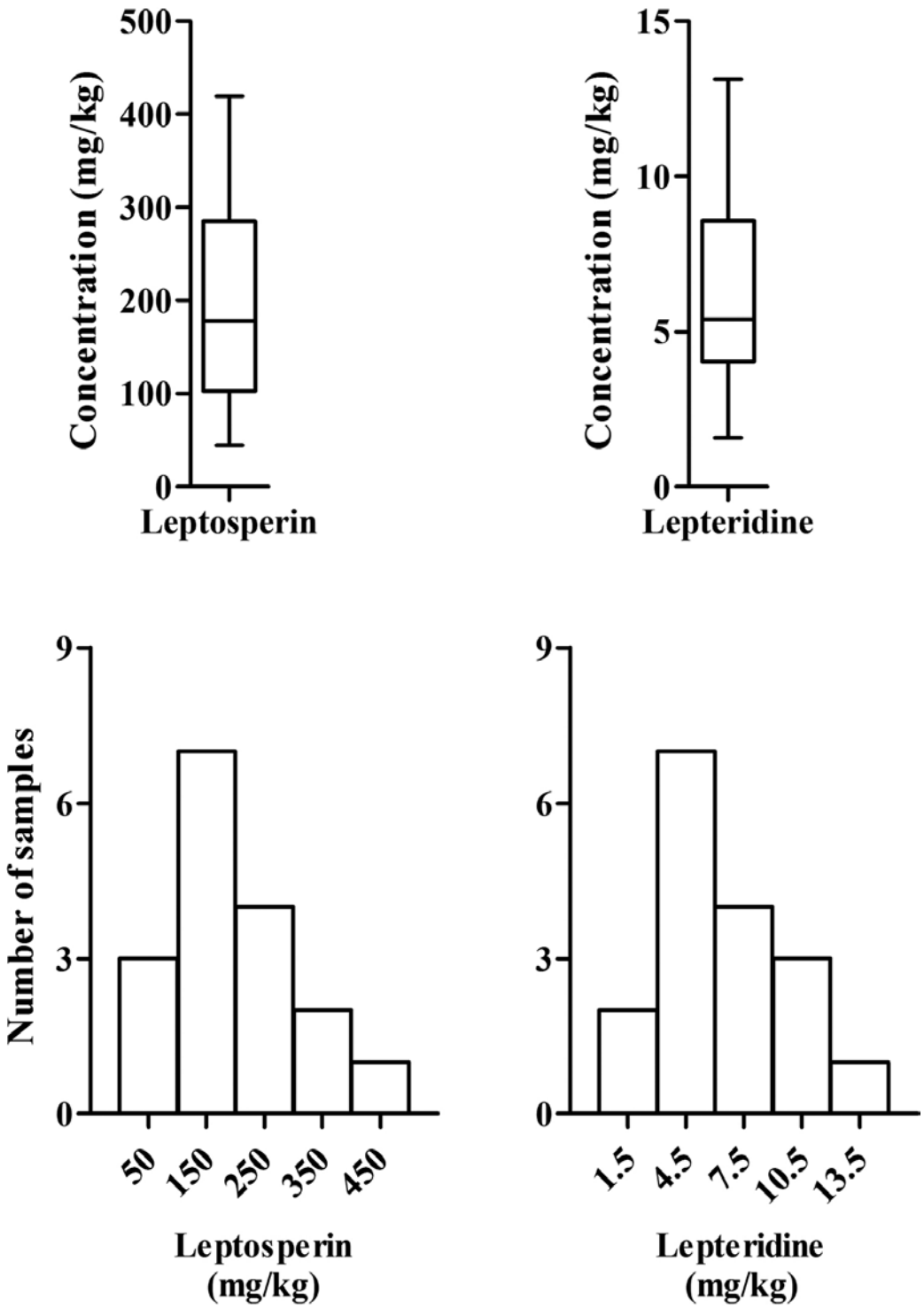

Figure 8. Statistical distribution of Leptosperin and Lepteridine in commercial manuka honeys.

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The relationship of Leptosperin and Lepteridine with the associated fluorescence for the commercial honeys is illustrated (**Figure 9**). For the purposes of this discussion, the minimum accepted concentration of Leptosperin is 100 mg/kg, and Lepteridine 4 mg/kg, these concentrations relate to MM1 (2000 RFU) and MM2 (500 RFU) in a honey matrix. RFU is an arbitrary unit and varies between fluorometers. These lower acceptable levels of these four parameters are illustrated in **Figure 9**.

Honey samples 1 and 2 do not meet the criteria for chemical concentration of either Leptosperin or Lepteridine, and fluorescence profile of both honeys did not meet the lower threshold. Honey sample 3 Leptosperin and Lepteridine concentration was 102 and 3.37 mg/kg, respectively. The fluorescence signature of this sample was slightly less than the threshold, and most probably was not wholly or predominantly harvested from *L. scoparium*.

When assessing honeys that are close to the lower threshold, it is appropriate to consider multiple characteristics and accordingly honey samples 4 and 6 are considered accepted despite the MM2 fluorescence being in the order of 450 RFU rather than 500 RFU.

Therefore, in this commercial set of manuka honeys, three out of 17 samples did not display the fluorescence characteristics or contain the concentrations of the key markers that would be expected to be encountered in an authentic manuka honey. Rapid assessment by fluorescence would have identified these three samples as requiring a full analytical workup, and allowing the balance of samples to be retailed as manuka honey.

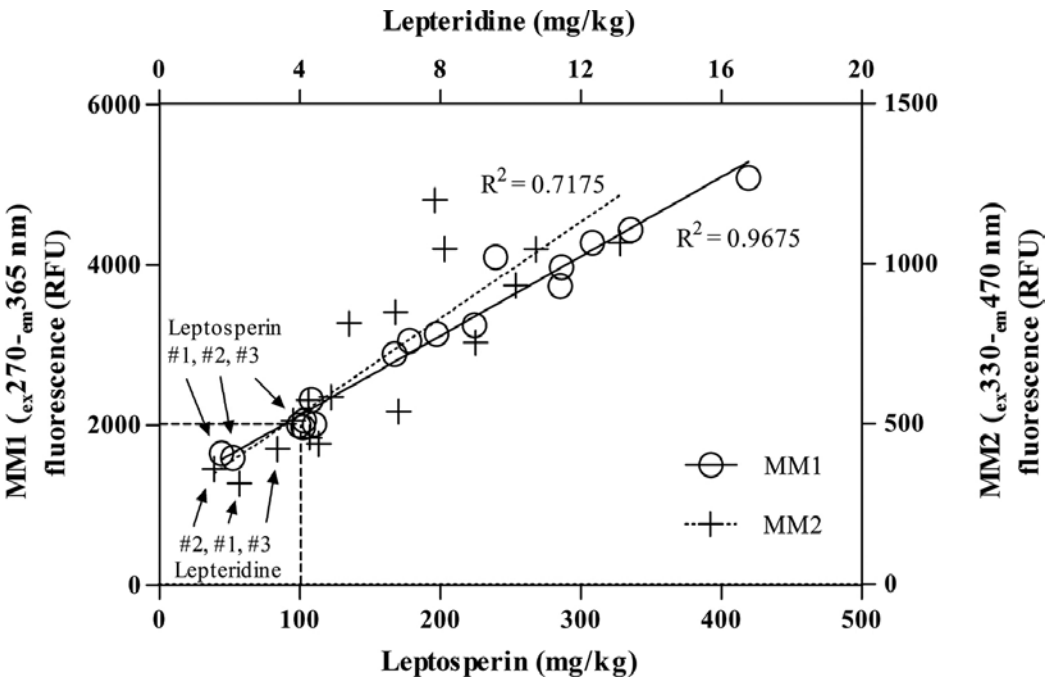

Figure 9. Commercial manuka honey samples Leptosperin and Lepteridine concentrations in relation to MM1 and MM2 fluorescence.

4. Conclusions and applications

Fluorescence analysis is a novel technique to determine manuka honey authenticity. Two unique compounds have been found in manuka honey, Leptosperin and Lepteridine, and these compounds are responsible for the MM1 and MM2 fluorescence described in the honey. In New Zealand, Leptosperin and Lepteridine are present only in *L. scoparium* nectar and therefore, these compounds are reliable chemical markers for manuka honey. However, the concentrations of these compounds do not predict DHA and MGO concentrations in a honey.

Fluorescence spectroscopy is a rapid technique with high throughput, and relatively simple fluorescence screening assessments are gaining increasing attention in food processing systems. Technology for assessing fluorescence is developing rapidly and handheld fluorometers are available. A handheld fluorometer could be used in the field by beekeepers, alternatively in market by retailers and may be of use to regulatory authorities.

Fluorescence assessment of manuka honey is an independent method separate from liquid chromatography coupled to detectors such as DAD or mass spectrometry. The use of two sets of wavelengths in combination, which can be screened simultaneously, adds robustness to this analysis.

Therefore, analysis of the MM1 and MM2 wavelengths is an efficient way of screening New Zealand honeys to ensure that attribution of floral source is appropriate and manuka honeys are wholly or predominantly sourced from *L. scoparium*, and these honeys that do not display characteristics of manuka honey are not inappropriately labelled.

5. Analytical techniques

5.1. Fluorescence spectroscopy

Honey fluorescence was analysed by scanning fluorescence spectroscopy according to methods described previously [36]. Honey samples were diluted with distilled water to 2% w/v, and loaded as 100 μL aliquots into a flat-bottom microplate (Optiplate™-384, black). Fluorescence measurements were carried out on a Gemini EM Dual-Scanning Microplate Spectrofluorometer (Molecular Devices Inc., Sunnyvale, CA, USA) operated with the SoftMax® Pro software. A fluorescence top read setting with automatic calibration and sensitivity at an ambient temperature was adopted for analysis at both MM1 and MM2 marker wavelengths. Fluorescence intensity was expressed as arbitrary units, in this case relative fluorescence units (RFU).

5.2. HPLC

Leptosperin, Lepteridine, methyl syringate and 2-methoxyacetophenone concentrations were quantified on a Dionex Ultimate™ 3000 reverse-phase HPLC system (Thermo Fisher Scientific, New Zealand) with diode-array detection (DAD).

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Honey samples were diluted 1 in 5 with 0.1% v/v formic acid. The injection volume was 3 μL. Separation was carried out on a Hypersil GOLD column (150 × 2.1 mm, 3μm particle size) by gradient elution at a constant flow rate of 0.200 mL>:in. The binary mobile phase consisted of 0.1% formic acid (Solvent A) and 80:20 acetonitrile:Solvent A (Solvent B). The gradient elution programme was as follow: initial (5% B, held 2 min), 7 min (25% B), 14 min (50% B), 16 min (100% B, held 3 min), 19 min (5% B, held 1 min) and 20 min (5% B, held 10 min). The column was thermostatically controlled at 25°C. Leptosperin, Lepteridine, methyl syringate and 2-methoxyacetophenone were monitored at 262, 320, 280 and 250 nm, respectively.

Data acquisition and peak integration were performed with Thermo Fisher Scientific™ Dionex™ Chromeleon™ 7.2 Chromatography Data System (CDS) software. The compounds of interests were quantified using external calibration curves of respective chemical standards based on integrated measurement of peak area.

5.3. UPLC and fluorescence methods

Honey samples (0.5 g) were weighed into a polypropylene extraction tube and solubilised in 9.5 mL of 10% acetonitrile containing 0.1% formic acid in Type 1 water by shaking and ultrasonic agitation. After centrifugation to remove particulates, an aliquot was diluted a further fivefold for analysis by ThermoFisher Ultimate-3000 UPLC with an RS fluorescence detector ($_{ex}$264nm–$_{em}$365nm), using a Waters XSelect HSS T3 C18 column (2.1 × 30 mm, 2.5 μm particle size). Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in methanol. The elution gradient started at 8% B (92% A) and increased to 100% B over 10 min before equilibration in 92% A for 3 min. Leptosperin was quantified against a synthetic standard.

For gross honey fluorescence analysis, 250 μL of the 20-fold diluted extract used for the Leptosperin analysis was added to the well of a fluorescence-grade 96-well plate and the gross fluorescence of each sample measured at MM1 using a SpectraMax i3 (Molecular Devices LLC, Sunnyvale CA, USA).

5.4. DHA and MGO

Concurrent analysis of DHA and MGO was carried out on a Dionex UltimateTM 3000 reverse-phase UPLC-DAD system (Thermo Fisher Scientific, New Zealand) following derivatisation with *O*-(pentafluorobenzyl) hydroxylamine hydrochloride (PFBHA). Honey samples were prepared in distilled water at a 1:20 w/v ratio. The samples were thoroughly mixed and incubated at 50°C for 1 hour to allow complete dissolution of any sugar crystals.

The derivatisation procedure was carried out according to methods developed by Kato et al. [41] with some modifications. A 2% stock solution of PFBHA was prepared in 0.1 M citrate buffer adjusted to pH 4 with NaOH (1 M). A working solution of PFBHA derivatising reagent was prepared consisting of 7:2:1 LC-MS grade acetonitrile:distilled water:PFBHA stock solution, and added to the honey samples at a 5:1 v/v ratio. The PFBHA:honey mixture was incubated at 50°C for 1 hour and cooled to room temperature.

A 5 μL aliquot of the derivatised sample or standard was injected into the UPLC-DAD system. Separation was carried out by gradient elution on a Hypersil GOLD column (100 × 2.1 mm, 1.9μμm particle size) at a constant flow rate of 0.700 mL/min. The mobile phase consisted of 0.1% v/v aqueous formic acid (Solvent A) and LC-MS grade acetonitrile (Solvent B), and the gradient elution programme was as follows: initial (B 20%, held 0.6 min), 1.3 min (B 70%), 3 min (B 100%, held 0.5 min) and 4 min (B 20%). The column was thermostatically controlled at 50°C. Dihydroxyacetone was monitored at 214 nm and MGO at 246 nm.

Data acquisition and peak integration were performed with Thermo Fisher ScientificTM Dionex™ Chromeleon™ 7.2 CDS software. Honey DHA and MGO were quantified using external calibration curves generated from the DHA and MGO working standards by linear regression of peak area against concentration.

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Chapter 6

Rheological Properties of Honey in a Liquid and Crystallized State

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Additional information is available at the end of the chapter

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Abstract

The rheological properties of honey are discussed separately for liquid and crystallized honey. The research methods used in both cases are characterized. The basic mathematical models are shown, which describe the viscosity of honey in its liquid form depending on temperature and water content. In the case of crystallized honey, the rheological properties were linked to morphological features and crystalline phase content. Results of characteristic experiments are presented, obtained during the shearing of crystallized suspension, that is, crystallized honey. Among other items, the dependency of equilibrium stress on shear rate, apparent viscosity on crystalline phase content, hysteresis loops as evidence that honey in its crystallized form is a rheologically unstable fluid. Results of measurements under forced oscillation conditions are included and compared with results of rotational measurements. It was shown that the research method influences the obtained results of rheological studies.

Keywords: viscosity, consistency, crystalline structure, rheology properties, cream honey, water activity

1. Introduction

The rheological properties describe the behaviours of matter under tensions resulting from external forces. Each real matter, whether a solid, liquid or gas, strains when exposed to external forces. We distinguish elastic, plastic and viscous strains. The behaviour of elastic bodies is described by Hook's law and is characterized by its disappearance once external forces are taken away. Plastic strain is permanent and remains even after the external force is gone. A perfectly plastic body is called a Saint-Venant's body. Viscous strain, also known as flow, is characterized by a constant increase in strain under constant stress. Perfectly viscous

fluids are described by Newton's law, which may be presented as a linear relation between shear stress and shear rate:

$$\tau = \eta \cdot \dot{\gamma}. \quad (1)$$

The η parameter in the equation above stands for dynamic viscosity. All fluids, which do not fulfil Newton's law, are called non-Newtonian fluids. Generally, non-Newtonian fluids are divided into rheologically stable, rheologically unstable and viscoelastic. They demonstrate partially viscous, elastic and plastic properties. Rheology is tasked with the description of these properties.

The rheological properties of honey are analysed mainly within the aspect of fulfilling the basic production processes such as hydraulic transport, mixing, heating or batching [1]. Viscosity is additionally one of the parameters of quality assessment of the product [2]. In multiple published reports on the rheological properties of honey, there is a common observation that it is in fact a Newtonian fluid [3–6]. A few publications hint at the existence of a clear thixotropic effect, although it is only seen in certain types of honey such as heather honey or the Manuka honey from New Zealand [5, 7]. It needs to be stressed, however, that such reports are with regard to honey in its liquid state also known as strained honey. The parameters, which significantly influence the dynamic viscosity of the analysed product, are temperature and water content [5, 8, 9].

Bee honey is a concentrated aqueous solution of sugars. Due to this, most of the obtained types of honey undergo crystallization when in storage [10]. The crystallization process results directly from the chemical composition, as in almost all types of honey glucose are present in its supersaturated state [5, 11]. Melezitose can also undergo crystallization in honeys. The resulting solid phase is a glucose monohydrate, which has various geometrical forms in crystallization [5, 12, 13]. Honey after crystallization is called set honey and is a two-phase structure, semi-solid, which substantially varies in its properties from the liquid state—strained honey [5, 14]. As a result of crystallization, the organoleptic properties undergo significant changes, mainly the honey's texture and water activity [15]. Literature regarding the rheological properties of crystallized honey is surprisingly modest. There are a few studies, which are just starting to analyse the issue [13, 14, 16, 17]. These studies only identify the specific rheological properties of crystallized honeys, as one of the characteristics which change after the crystallization process.

This analysis is an attempt at the identification of the rheological properties of honey both in its liquid state and in its crystallized form. The performance of this task has forced an analysis of additional issues, which determine the rheological characteristics. These are the measurement of the amount of solid phase formed after crystallization of the honey and its morphological characteristics. These issues are relatively seldom analysed in literature [14].

2. Research methods used in the identification of the rheological properties of honey

Rheological measurements can be conducted using two different measurement techniques: rotational rheometers and capillary rheometers. Due to the speed, comfort of use

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and the possibility of measurement in wide spectrums of shear rate, rotational rheometers are in popular use at present. The used measurement systems are cone-plate, plate-plate or cylinder-cylinder in a Searle- or Couette-type rheometric flow. Modern-day rheometers are automated and allow obtaining information on the rheological properties relatively quickly, with relatively wide spectrums of shear rate in a precisely planned and repeatable way. The basic problem, which influences the choice of research method significantly, is the presence of the crystallized phase in the case of crystallized honey. The presence of crystals causes significant changes in the rheological properties, which in turn forces a choice of a proper measurement method. A wrong choice of measurement method for crystallized honey can result in unreliable results. As mentioned earlier, crystallized honey is a two-phase mixture, semisolid [5, 14]. It is a popular substance in the food industry. These can be products ready for consumption or half-finished products, which are undergoing processing in the form of solid-liquid mixtures [18]. Crystallized dispersions are also formed by metals in high temperatures. Semisolid media are usually characterized by non-Newtonic properties, and in order to identify them, complex rheological analysis methods are required [18–20].

Research methods regarding liquid and crystallized honeys are characterized below, separately. Additionally research aspects related to measurements of weight fraction and quantity morphological characteristics of the solid (crystallized) phase in crystallized honeys were noted.

2.1. Research methods used in the identification of the rheological properties of liquid honey

Liquid honey is a homogeneous fluid, a concentrated solution of sugars and other liquid substances. The majority of liquid honeys have characteristics of Newtonian fluids, which is why there are few limitations to research methods. From a researcher's point of view, the most beneficial measurement systems are the cone-plate or plate-plate ones. Such systems are easy to use and only a few millilitres of honey samples are required for rheological identification. The exchanging of the analysed medium in these systems is easy and the thermostating is satisfactory. Nevertheless, one may use the cylinder-cylinder measurement systems using the Searle or Couette flow. It is then necessary to have a larger amount of the pressure fluid and its exchange is more difficult. The identification of the viscosity of strained honey sample requires measurements to be conducted in at least a few or at best a few dozen measurement points, at which the shear stress for the assigned shear rates would be noted. The viscosity value is obtained by approximating the results to a linear function. The results of such an experiment are presented in **Figure 1**. Due to the fact that honey samples viscosity greatly depends on the temperature and water content, the values of these parameters are worth noting for every measurement. The viscosity value is a numeric coefficient in the obtained equation after the approximation of the experiment's results. In the example shown in **Figure 1**, the dynamic viscosity value is 12.95 Pas. Whether it is a Newtonian fluid is decided by the fact the points align themselves in a straight line. A fine measure of linearity (Newtonian properties) is the determination coefficient. If its value is greater than $R^2 > 0.95$ it may be stated that the fitting of the results to a Newtonian model is very good.

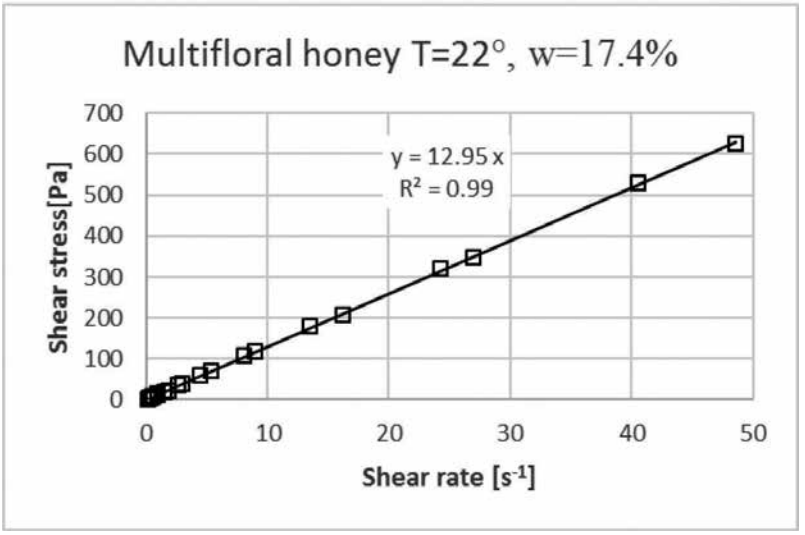

Figure 1. Method of determining the dynamic viscosity of liquid honeys.

A similar test can be conducted for any fluid of unknown rheological properties. It is the first effect of a rheological identification of a fluid. If the points do not align along a straight line, it serves as proof of the fluid being non-Newtonian, and a precise research methodology can be chosen.

There are numerous mathematical models used to describe rheological properties of honey in its liquid state [2, 21, 22]. Their main focus is on the description of the viscosity in the function of temperature. Arrhenius's equation is most often used for this purpose [8, 23]:

$$\eta = \eta_0 \exp\left(\frac{E_a}{RT}\right). \tag{2}$$

There is an opinion that this equation describes the dependency between viscosity and temperature relatively well, and the obtained results have an error margin no greater than 4.41% [23, 24].

In the literature analysing the issue, the William-Landel-Ferry (WLF) equation is also used to model the influence of temperature on honey viscosity [21]. This dependency uses glass-transition temperature and the viscosity in glass state to describe the dynamic viscosity of honey [22]. The mathematical equation is in the following form [21, 22]:

$$\ln\left(\frac{\eta}{\eta_g}\right) = \frac{-C_1(T - T_g)}{C_2 + (T - T_g)}. \tag{3}$$

It is also noted that the WLF dependency describes honey viscosity in the function of temperature very precisely, while this description is very sensitive to changes of the composition of the medium [21, 22]. The usage of the WLF model is relatively valuable in a general rheological analysis of honey and the identification of glass-transition temperature, which is made possible based on rheological measurements.

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The main drawback of the dependencies described above used to describe honey viscosity is the omitting of water content in the product. This is a parameter, which influences honey viscosity as significantly as temperature. This is why two-parametric models are used for practical purposes, which include both water content and temperature [1].

Dynamic measurements are also becoming more common in the analysis of rheological properties of honey [2, 22]. This also includes mixtures of honey and propolis as well as other food products [25]. The measurements are mainly connected with the identification of the value of the complex modulus G^*, storage modulus (elasticity) G' and viscosity modulus (loss) G'', phase angle δ and complex viscosity η^* in relations to the frequency [20]. The results are also presented in the form of graphs showing the changes of the elasticity modulus, the loss modulus and the complex viscosity in the function of yaw rate [2, 22]. There is no uniform opinion on the range of frequencies used in the measurements. The results are characterized by a generally linear dependency of both the storage modulus and the loss from frequency. The determined complex viscosity values in dynamic measurements do not always correspond to the dynamic viscosity. The measurements of the complex modulus G^* and the phase angle yield a uniform assessment of the rheological behaviour of the medium and determine the storage modulus (elasticity) G' and the loss modulus (viscosity) as simple dependencies [20]:

$$G' = G^* \cdot \cos\delta \tag{4}$$

$$G'' = G^* \cdot \sin\delta \tag{5}$$

The value of the phase angle δ is determined by the division of energies in the deformed medium into stored energy and energy lost to induce flow [20]. The closer the value of the angle is to $\delta = 90°$, the closer the properties of the substance are to a completely liquid state (Newtonian body). If $\delta = 0°$, the substance is completely elastic and is a hook's body [19].

2.2. Research methods used in the identification of the rheological properties of crystallized honey

As a matter of fact, measurements of the rheological properties of crystallized honey samples should be conducted solely by the use of rotational rheometers, additionally with a cylinder-cylinder measurement system. This is due to the fact that systems characterized by narrow openings generate significant errors in research because of the dimensions of the crystals. It is believed that the openings should have a linear dimension of at least three times the size of the largest crystal.

An additional effect, which needs to be taken into account in rheological studies of suspensions with a high concentration of solid phase such as honeys, is the unstable behaviour during shearing [19]. In the case when this influence is reversible, the fluids are thixotropic or antithixotropic [19, 20]. Irreversible changes in rheological properties of fluid are called rheodestruction or rheomalaxis [20]. This is connected with the destruction of the crystalline structure during shearing. The characteristic of rheologically unstable fluids requires the use of different research methods and additional rheological parameters. An additional and important aspect is the method of preparation and placement of the analysed medium in the

measurement system, as the activities connected with filling the measurement cylinders significantly influence the results of the analyses.

In the case of suspensions with a high-weight concentration of solid body, which present non-Newtonic behaviour, apparent viscosity is commonly used for rheological characteristic [20]. The apparent viscosity η' is defined as a relation between the value of shear stress and shear rate:

$$\eta' = \frac{\tau}{\dot{\gamma}}. \tag{6}$$

Apparent viscosity is the simplest and often used parameter of rheological characteristic of crystalline suspensions as well as other non-Newtonian fluids [19]. A graphic representation of changes in the apparent viscosity in the function of shear rate allows also to create a precise rheological characteristic of rheologically stable fluids.

In the studies of rheological properties of suspensions, which are in most cases rheologically unstable, equilibrium viscosity η_{eq} is used [19]. This parameter can be identified as the relation of equilibrium stress τ_{eq} obtained during shearing at a constant rate $\dot{\gamma}$ for a period of time long enough for equilibrium in the deformed system to occur:

$$\eta_{eq} = \frac{\tau_{eq}}{\dot{\gamma}}. \tag{7}$$

The representation of equilibrium viscosity or equilibrium stress in the function of shear rate is commonly used for rheological characteristic of suspensions [19]. Experiments are usually conducted with shear speed increasing in increments [20]. Equilibrium stress in crystallized honey samples is usually described using the Ostwald-de Waele model [14]:

$$\tau_{eq} = k \cdot \dot{\gamma}^n. \tag{8}$$

Another aspect of analysing the properties of semisolid sets related with their instability is the determination of behaviour in a closed cycle of shearing with increasing and then decreasing shear rate. By using a constant tempo of increase and then decrease of shear stress, characteristic changes in time are obtained in the form of a hysteresis loop [20]. This constitutes a traditional quality test for the occurrence of thixotropia [19]. By repeating an identical shear cycle after a certain amount of time of the medium remaining dormant, an answer can be obtained to the influence of time on the rebuilding of the internal structure of the crystalline suspension. Conforti et al. used such a cycle: with an increasing shear rate from 0 to 320s^{-1} for 1.5 min, holding for 2 min at $\dot{\gamma} = 320s^{-1}$ and then decreasing with an identical shear tempo, for the rheological characteristic of honey samples in their crystallized state [13]. It was proven that all analysed honey samples presented with a hysteresis loop whose shape was determined by the crystalline structure of the analysed media.

Measurements which enable us to obtain a hysteresis loop need to be performed with caution, for the cylinder slip not to occur causing the syneresis effect [19, 20]. It needs to be mentioned that the possibility to compare the hysteresis loop is available only when the loops are obtained in an identical shear cycle with a constant increase of deformation rate and its consecutive decrease. Additionally, there is a strong influence of the human factor related to the method of introducing the sample into the measurement system of the rheometer [12].

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The thixotropic effect is also analysed in the microstructural context, as stress changes during shearing are related with the transformations occurring in the internal structure of the fluid. The scalar value κ also called the structural parameter is used for this purpose [19]. Then, the thixotropic behaviour of the substance can be described using two constitutive equations:

$$\tau = f(\dot{\gamma}, \kappa); \tag{9}$$

$$\frac{d\kappa}{dt} = g(\dot{\gamma}, \kappa). \tag{10}$$

When the equilibrium stresses τ_{eq} are reached, that is, the shear rate of the structure equalsits rebuild rate, then the growth $\frac{d\kappa}{dt} = 0$ and the structural parameter has the equilibrium value $\kappa = \kappa_{eq}(\dot{\gamma})$. Eq. (**9**) has the form [19]:

$$\tau = f[\dot{\gamma}, \kappa_{eq}(\dot{\gamma})] = \tau_{eq}(\dot{\gamma}). \tag{11}$$

This is the equilibrium flow curve, which as mentioned above for crystallized honey samples can have the form of relation (**8**). Nevertheless, one can find reports in the literature on the usage of the structural parameter defined in a different way [4, 20].

The complement of the empirical methods of the rheological analysis of crystallized honey samples is studies conducted using a dynamic rheological test. Such techniques are very useful to measure properties of suspensions, in which complex interactions between the ingredients take place. By determining the conditions of the decay of structures forming such sets during shearing, it is possible to obtain a precise rheological characteristic [20]. A classic method in this regard is to use oscillation measurements to identify the influence of temperature and pH of the environment on the blood coagulation process [26].

The values measured in dynamic measurements are usually complex modulus G^* and the phase angle (Eqs. (**8**) and (**9**)). Based on these two parameters, it is possible to conduct a uniform assessment of the rheological behaviour of the medium. Viscoelastic media are additionally characterized by a parameter called complex viscosity, which is defined as the ratio of the complex modulus to the angular frequency of oscillation [20]:

$$\eta^* = \frac{G^*}{\omega} \tag{12}$$

Attention is paid to the fact that between the complex and dynamic viscosity, there is a dependency called the Cox-Merz dependency [20]:

$$\eta^* = \eta\big|_{\omega=\dot{\gamma}}. \tag{13}$$

Lazaridou et al. stated that in the case of Greek honey in liquid form, the value of dynamic viscosity is generally greater than that of complex viscosity [2]. Ferguson and Kembłowski (1991) noted that the Cox-Merz dependency has a limited range of use in the case of suspensions due to the structural differences of these fluids while dormant and while in set flow. In the case of semisolid food products, the Cox-Merz dependency is modified to the form of [20]

$$\eta^* = C\,\eta^{\alpha}\big|_{\omega=\dot{\gamma}}. \tag{14}$$

There are, however, no data whether the above-mentioned rule can be used for crystallized honey.

2.3. Research methods used to measure the amount of solid phase in crystallized honey

Reports on the amount of solid phase formed in honey are relatively modest. Existing data point to this value being approximately 15% [12]. Meanwhile, this parameter defines, at a very basic level, the rheological properties of crystallized honey. It would seem that the answer to this question can be obtained by simply comparing the solubility of glucose in water (saturation concentration) at a given temperature with its content in the product. The result of this comparison is not, however, so obvious. Glucose can crystallize in an anhydrous form and as a monohydrate [5]. Data on the solubility of anhydrous glucose point to its saturation concentration (in an aqueous single component solution) at a temperature of 25°C being approximately 60% [27]. The solubility of glucose monohydrate is lower and in these conditions amounts to slightly above 50% [27]. Zamora and Chirife assume that the value of saturation concentration of glucose in water at 25°C is 103.3 g of glucose per 100 g of water [28]. By relating the glucose content to water content for various honeys, we can obtain a glucose concentration level of 1.5–2.5 g of glucose/g of water [28]. Glucose in almost all types of honey is present in a supersaturated state. Fructose, despite the fact that its content is higher in most honeys than that of glucose, never reaches its saturated state, which is 405.1 g per 100 g of water [5, 27, 28]. Nevertheless, there is an influence of fructose on the crystallization process of glucose and it is necessary to perceive honey as a ternary set of water, glucose and fructose. The results of studies by Lothrop and Kelley regarding the equilibrium of such sets show that they are sufficiently complex [5]. It is generally known that a high addition of fructose reduces the tendency to crystallize. Own research of model aqueous solutions of glucose and fructose allowed to make visible the significant influence of fructose on the crystallization process of glucose [29]. The increase of fructose concentration in a supersaturated glucose solution extended the time of crystallization changes the morphology of the crystals formed and reduces the amount of crystallized glucose [29]. Measurements using computer imagery analysis allowed to show that the formed crystals of glucose monohydrate in the presence of high concentration of fructose within the solution are characterized by larger size in comparison to crystals obtained from pure glucose solutions. There is, additionally, a linear increase in absorbance in the infrared spectrum of glucose suspension under the influence of an increased mass fraction of solid phase [12]. Lupano analysed changes in the absorbance of honey samples during crystallization at a wavelength of λ = 660 nm and stated that they depend on the crystallization temperature [30]. Conforti et al. searched for a dependency between the absorbance determined at λ = 660 nm, for various types of honey samples and the water content and parameters determined on the basis of chemical composition. The results of these comparisons did not yield uniform results [13].

A standard approach to determining the amount of solid phase, which melts in the mixture, is by using differential scanning calorimetry (DSC). In the case of honey, there are several reports on the use of DSC to analyse crystallized honey samples [2, 14, 17, 22, 30]. The results of these analyses show unarguably that DSC allows for a perfect identification of the glass-transition temperature together with the caramelization temperature and other changes occurring in carbohydrates in high temperatures [2, 14, 22]. Lupano reports that in the range of 20–50°C, changes occur on the DSC thermograms, which strongly depend on the conditions in which the crystallization of honey takes place and are characterized by a low value of enthalpy with a significant standard deviation of results [30].

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Crystallization changes significantly the way of binding water within a product. Growth in water activity caused by the crystallization in honey samples is from approximately 0.012 even to as much as 0.12 with an average value of 0.027 [12]. Identical results obtained for 49 Argentinian honey samples yielded values from 0.014 to 0.056 with an average value of 0.034 [28]. Glieter et al. showed that the increase in water activity after crystallization depends on the origin and for nectar honey samples it has the value of approximately 0.04 and for honeydew honey samples of 0.02 [15]. Nevertheless, the literature lacks in a clear explanation of the factors determining the increase in water activity in honey samples after crystallization. Bhandari and Bareyre [31] showed on model glucose solutions that the dissolution of glucose monohydrate lowers the water activity proportionally to the weight of dissolved crystals. Basing on this, a conclusion was reached that through changes in water activity, the amount of crystallized solid phase can be determined. Own research conducted under similar conditions, but using honey samples, allowed to show that the explanation is not that obvious.

The measurement of mass fraction of solid phase in crystallized honey samples is possible using near-infrared spectroscopy (NIR). Near-infrared spectroscopy is an effective measurement technique enabling the conducting of complex analyses of the crystallization process [32]. The NIR spectroscopy is especially effective in analysing food product sets, which contain water. Own research showed a linear increase in the absorbance with an increase in the mass fraction of the crystalline phase in aqueous glucose suspensions. By using NIR spectroscopy, it is possible to analyse other occurrences in honey samples during crystallization [32].

The analysis procedure for determining the mass fraction of crystallized phase in crystallized honey comprises two stages [12]. The first stage is to determine the calibration equations using preparations with a known mass fraction of crystalline phase in a given honey samples (different honey samples show different absorbance values). In the second stage, measurement is possible of the mass fraction of crystalline phase in crystallized honey. The identification of solid phase has to be conducted for a wavenumber of $\nu \approx 4467\ \mathrm{cm}^{-1}$ [12]. This results from the fact that for this value of the wavenumber there is an isobestic point in glucose solutions. In an isobestic point, the absorbance values of glucose solutions have a constant value, which is the same as the absorbance of water and does not depend on the concentration of glucose in the aqueous solution. For the value of $\nu \approx 4467\ \mathrm{cm}^{-1}$, there occurs one of the local extremes on the differential spectrums of liquid and crystallized honey samples [12]. Fructose solutions do not have at this point an isobestic point. Nevertheless, the absorbance values of aqueous fructose solutions for $\nu \approx 4467\ \mathrm{cm}^{-1}$ are also close to absorbance of pure water. By using this information, it is possible to state that the increase of absorbance in crystallized honey samples for a wavenumber of $\nu \approx 4467\ \mathrm{cm}^{-1}$ is connected only to the presence of solid phase in the form of glucose monohydrate within the honey [12].

2.4. Quantitative measurement of the morphology of a crystalline structure

Crystals formed in honey during crystallization are most commonly presented as photographs made using ordinary optical microscopes [13, 14, 17, 30]. Unfortunately, such images are not very clear and troublesome in interpretation and in computer editing using software

for computer-aided image analysis. The literature suggests the existence of images of such crystals made in polarized light [33]. They enable a relatively effective presentation of morphology of the crystalline phase in the honey samples. A detailed analysis of the crystalline structure of honey samples under birefractive interferometry allowed to prove that it is an extremely effective research technique, as glucose monohydrate crystals are characterized by optical birefringence [12]. Measurements of the morphology of the crystalline structure conducted based on images obtained under birefractive interferometry in transition lighting in the so-called black background using a bipolar PI interfero-polarizing microscope are very effective [12, 34]. Takes is to place a drop of honey between two microscope slides. Due to the need for sharp images of the crystalline structure, the thickness of the medium layer cannot exceed 0.1–0.2 mm. It is difficult under these conditions to photograph the crystalline agglomerates occurring in honey samples, as they have a higher thickness. In order to minimize the phenomenon of interfusing of the crystals in own research, a method was devised of displaying the crystals through introducing a thin layer of crystallized honey onto the liquid honey. In this way, it was possible to minimize the occurrence of interfusion of crystals in images. Observations can be conducted with a magnification of approximately 150× using a charge-coupled device (CCD) camera. **Figure 2** shows two sample images of crystallized rape and buckwheat honey [12].

Quantity characteristics of the morphology of the crystalline structure of crystallized honeys can be obtained through determining the distribution of the number of crystals in reference to a characteristic dimension, for example, the maximum diameter (maximum linear dimension of crystals). In order to provide representative nature of the conducted analyses, a sufficiently large population needs to be taken into analysis, for example, one composed of 2000 crystals. The analysed images should be chosen at random. It was shown that crystals in crystallized honey samples demonstrate empirical distribution of exponential character in relations to maximum diameter:

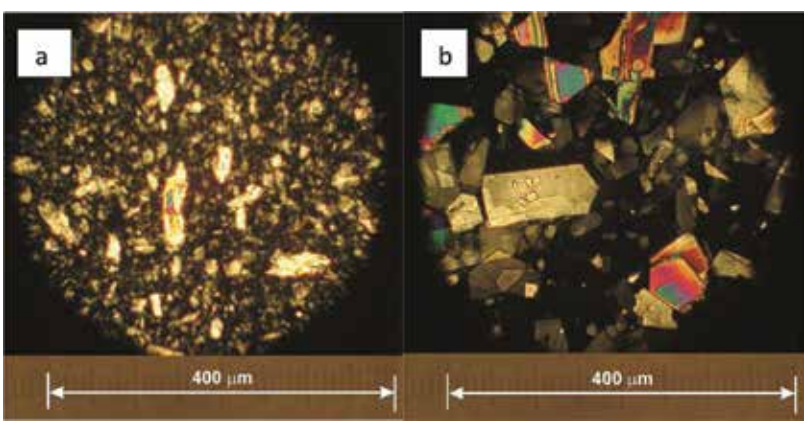

Figure 2. Images made under birefractive interferometry showing the structure of crystallized honey samples: (a) rape honey and (b) buckwheat honey [12].

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$$N(d_{\max}) = \lambda \cdot \exp(-\lambda \cdot d_{\max}) \tag{15}$$

Due to the fact that exponential distribution is characterized by one parameter, there is a possibility of quantitative characteristic of the morphological crystalline structure the analysed honeys through a comparison of the λ values [12].

3. Rheological properties of liquid honey

As mentioned earlier, liquid honey has the properties of a Newtonian fluid with a high viscosity value, which strongly depends on temperature. **Figure 3** shows two sample flow curves obtained through rotational measurements (which in this case are straight lines—Newtonian fluid) of honey at a temperature of 298 and 308 K. A 10° increase in temperature caused a decrease of viscosity from 12.95 to 5.52 Pas, which is over 57%. It is worth noting that this viscosity value is a few (a few dozen) thousand times higher than that of water, which is 0.001 Pas. By expanding the range of temperatures, it can be easily shown that its influence in the lower values is even greater. **Figure 4** shows the results of viscosity measurements of buckwheat honey with a water content of 18.1% at a temperature range of 268–295 K. The results of this experiment can be approximated to the exponential curve, whose equation is shown in **Figure 4**.

Nevertheless, water content also significantly influences the viscosity of honey. Oppen and Schuett as early as in 1939 published an equation, which describes the relations between the viscosity logarithm and water content [35]:

$$W = (62,500 - 1567)\,[\,T(\log \eta_T + 1) - 2287(313 - T). \tag{16}$$

Junzheng and Changying developed a fairly simple dependency based on empirical studies [1]:

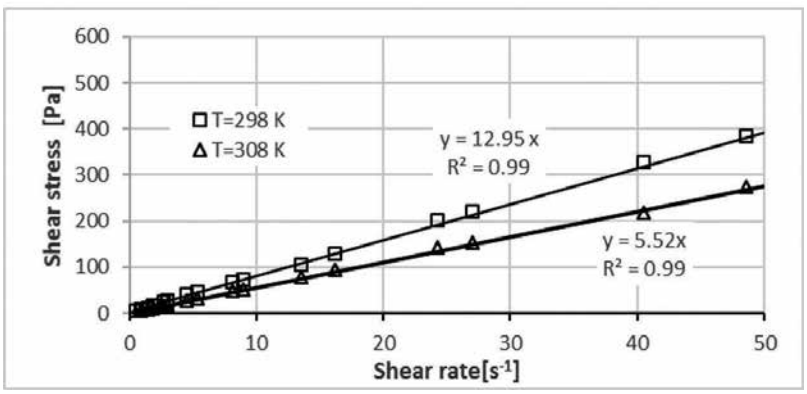

Figure 3. Sample results of rheological measurements—flow curves of multifloral honey w = 17.6% at a temperature of 298 and 308 K.

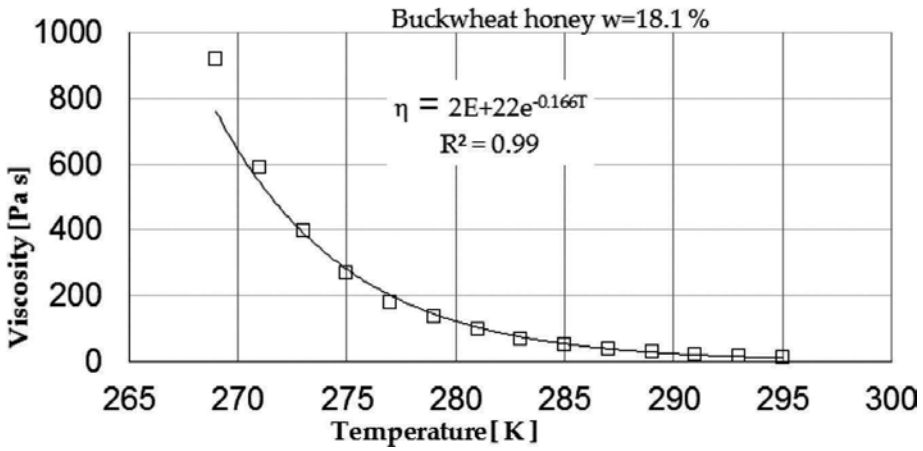

Figure 4. Dependency of buckwheat honey samples viscosity on temperature in the range of 268–295 K.

$$\eta = 14.2 \cdot 10^3 \cdot \exp(-0.31 \cdot w - 0.085 \cdot t). \tag{17}$$

A similar equation was used to describe the viscosity of Spanish honeys [9]:

$$\eta = 19.2 \cdot 10^3 \cdot \exp(-0.3 \cdot w - 0.087 \cdot t). \tag{18}$$

Eqs. (**17**) and (**18**) were formed for a relatively high water content percentage, which is in the range from 17.07 to 34.06% and a narrow range of temperature in Celsius [1, 9]. They show that it is relatively easy to describe the viscosity of liquid honeys—taking into account both the temperature and the water content.

Own research conducted on a few hundred samples of Polish honeys for a wide range of temperatures from 260 to 330 K allowed to determine that there is a dependency between water content and temperature expressed in absolute terms [29]:

$$\mu = 1.72 \cdot 10^{22} \cdot \exp(-38.363 \cdot W - 0.1398 \cdot T) \tag{19}$$

The difference in the values of numeral coefficients of the equation above in relations to dependencies (17) and (18) is mainly the results of the usage of temperature expressed in absolute terms and expressing water content by a mass fraction. A graphic illustration of the above-mentioned dependency is shown in **Figure 5**. It is interesting that for a temperature below 0°, all types of honey show high viscosity exceeding 1000 Pas.

The dependencies presented above (17–19) can be accepted as approximated mathematical models of viscosity of liquid honey samples. It needs to be kept in mind that honey shows changeability related to various environmental factors. However, for technological purposes, these dependencies allow for sufficient approximation of the viscosity value in relations to temperature and water content. These relatively simple relations allow to determine

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the value of honey samples viscosity for a wide range of temperatures and water content and to perform calculations connected with hydraulic transport, mixing or heating of the honey.

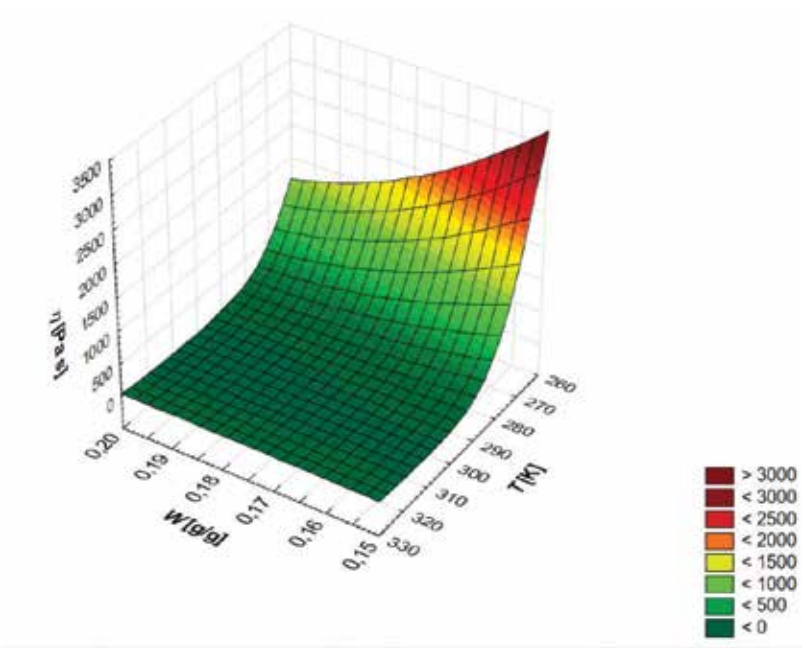

Figure 5. Relation of the viscosity of honey samples to temperature and water content [29].

To finish the discussion on the rheological properties of liquid honeys, attention must be paid to the fact that the measurement results in a dynamic rheological test are similar to rotational measurements. The values of complex viscosity of the analysed media are similar to the values of dynamic viscosity and the relative differences between the average values of dynamic viscosity and complex viscosity do not exceed 10% [12].

4. Rheological properties of crystallized honey

In the case of crystallized honey, the task of determining the rheological properties is more complicated. Honey is not a homogeneous body, it does not show Newtonian properties and additionally it becomes solid after longer periods of storage. In order to analyse such a medium, cylinder-cylinder systems seem to be the most appropriate. Even the filling of the measurement system with crystallized honey can be problematic, as the block needs to be crushed, which at a temperature below 20°C can be difficult. The method used to this end can later influence the results of the experiment, so it needs to be done in a repeatable fashion. Such a problem does not occur in the case of creamed honey, which is obtained (to put in plainly) by mixing of the crystallizing mass. Rheological properties of crystallized honeys can be influenced by the mass fraction and shaping (morphology) of the crystalline phase apart from temperature and water content.

The crystalline structure of different types of honey can vary significantly, which is a result of differences in chemical composition—mainly the content of glucose, fructose and water [36]. The morphology of crystals is also significantly influenced by crystallization conditions. **Figure 6** shows images of the crystalline structure of three types of honey: rape, multifloral and buckwheat. Even a superficial quality assessment conducted based on visual data allows to identify significant differences. The results of sample measurements, which allow to quantitatively characterize the populations of crystals of the individual types of honey samples, are shown in **Figures 7–9**. Rape honey is characterized by the largest crystal fraction with a d_{max} of <10 μm [12]. The multifloral honey has a large crystal fraction of $10 < d_{max} < 30$ μm in diameter [12]. Buckwheat honey has a large number of crystals with the dimensions of $30 < d_{max} < 70$ μm [12]. The numerical distribution of buckwheat honey crystals clearly distinguishes it from the other honeys through a characteristic local extreme for the $30 < d_{max} < 35$ μm fraction and is close to the results obtained by Mora-Escobedo et al. for the Mexican tajonal honey. The obtained results using the maximum diameter characterize the morphology of the crystalline structure more clearly than using the crystals' surface area [14]. Distributions characterizing the population of crystals have an exponential character and can be described unambiguously using the λ-parameter.

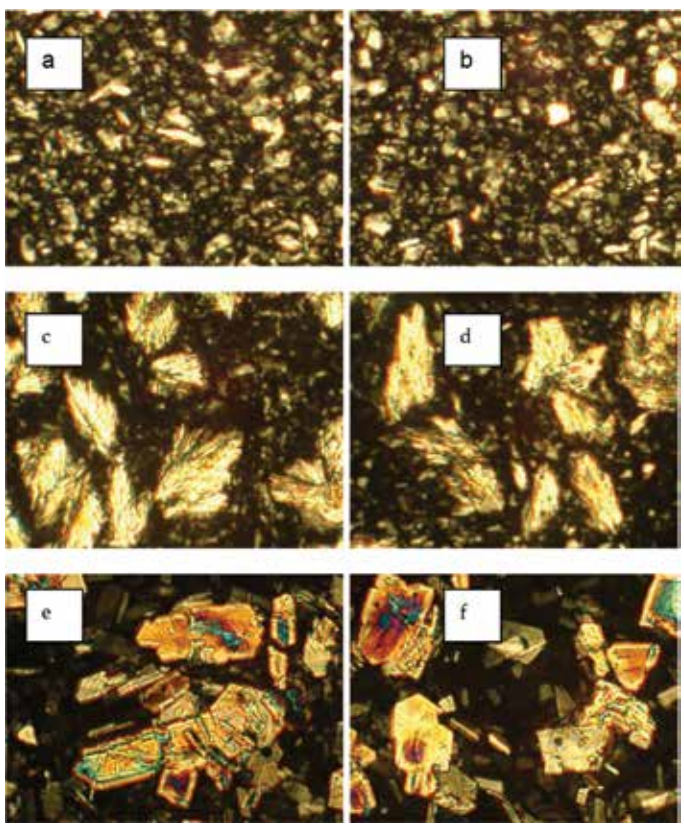

Figure 6. Images showing the morphology of the crystalline structure of honeys samples: (a) and (b) rape honey, (c) and (d) multifloral honey, (e) and (f) buckwheat honey.

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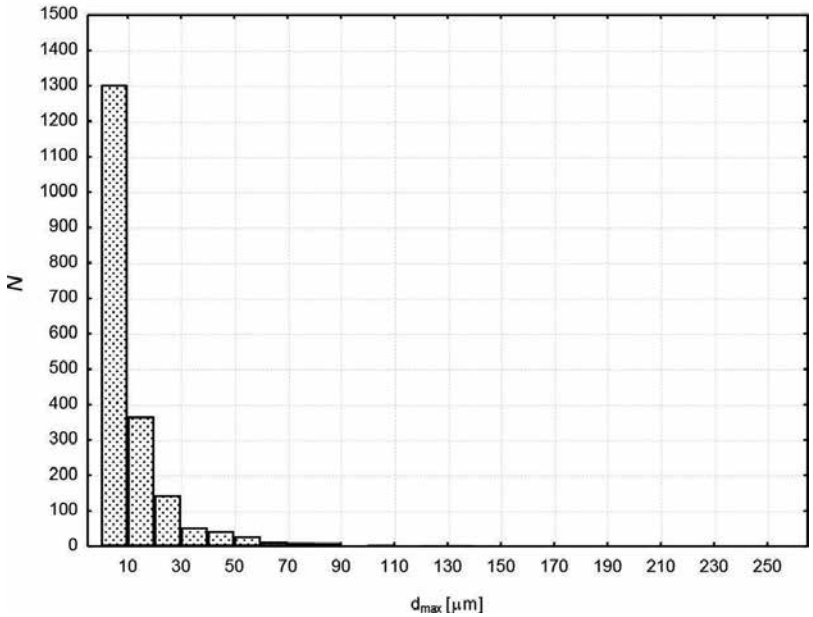

Figure 7. Numerical distribution of the population of 2000 crystals identified in rape honey samples according to maximum diameter.

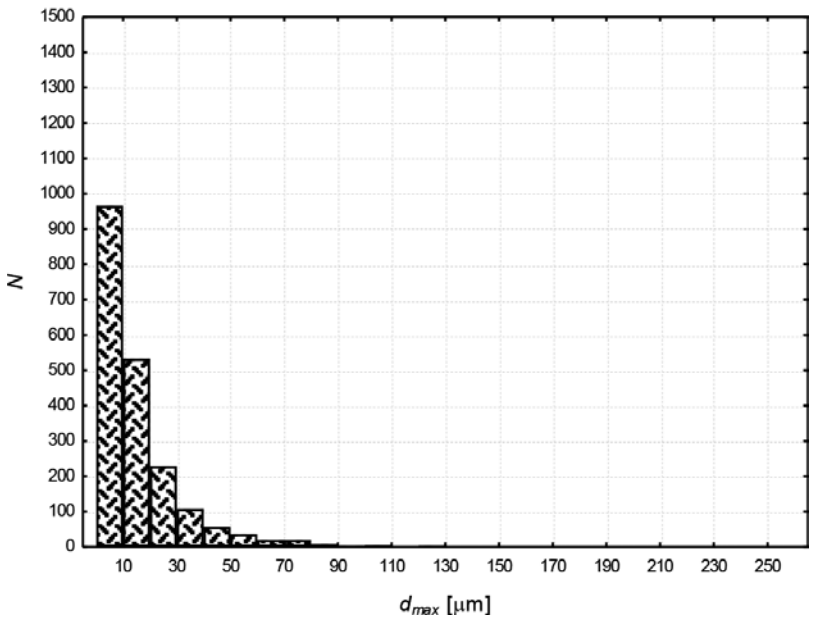

Figure 8. Numerical distribution of the population of 2000 crystals identified in multifloral honey samples according to maximum diameter.

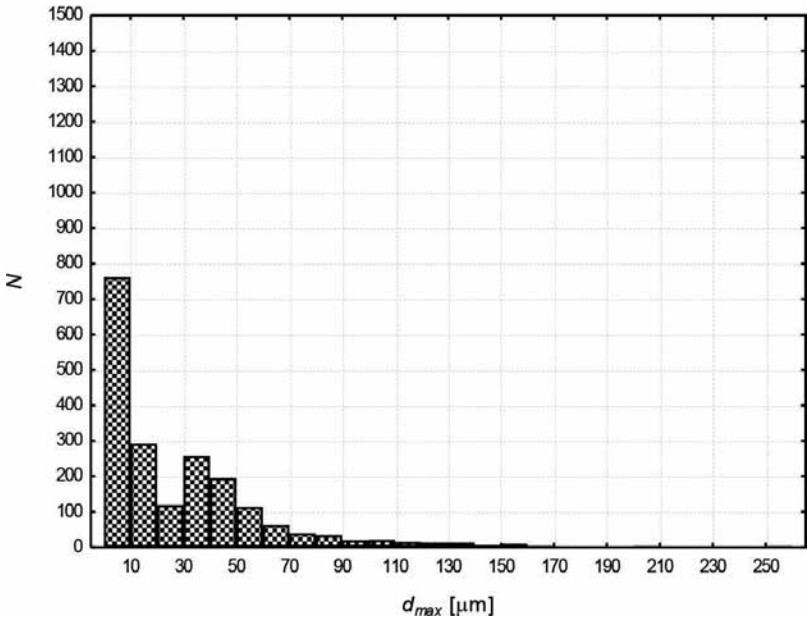

Figure 9. Numerical distribution of the population of 2000 crystals identified in buckwheat honey samples according to maximum diameter.

It is best to begin the rheological characteristic of crystallized honeys from the presentation of equilibrium flow curves (**Figure 10**). As a reminder, the equilibrium flow curve is obtained through assigning equilibrium stress values to shear rate values. The equilibrium stress values are read after stabilizing at a constant level with shearing at a constant shear rate. Next, the value of shear rate is increased in increments and the measurement is repeated.

Based on the flow curves shown in **Figure 10**, the influence of morphology of the crystalline structure on the rheological properties of the analysed suspensions can be estimated. It needs to be mentioned, however, that the content of solid phase in these media was rape 18.2%, multifloral 18.5% and buckwheat 19.2%. The rape honey curve is located the highest and the stress increases at the fastest rate in relation to the increase in shear rate despite the fact that the solid phase content is not the highest. Multifloral honey is characterized by a flow curve located below the rape honey, while the flow curve of buckwheat honey is located below the previous two [12, 36].

A large amount of small crystals causes a significant increase of the texture coefficient and causes the stress in the suspension to increase quickly with the increase of shear rate. Crystallized honeys with large and flat crystals show lower values of the texture coefficient as well as apparent viscosity [12]. The flow curves shown in **Figure 10** can have the following dependencies assigned to describe the apparent viscosity:

$$\text{rape honey} \quad \eta' = 122.07 \times \dot{\gamma}^{-0.604} \tag{20}$$

$$\text{multifloral honey} \quad \eta' = 56.54 \times \dot{\gamma}^{-0.466} \tag{21}$$

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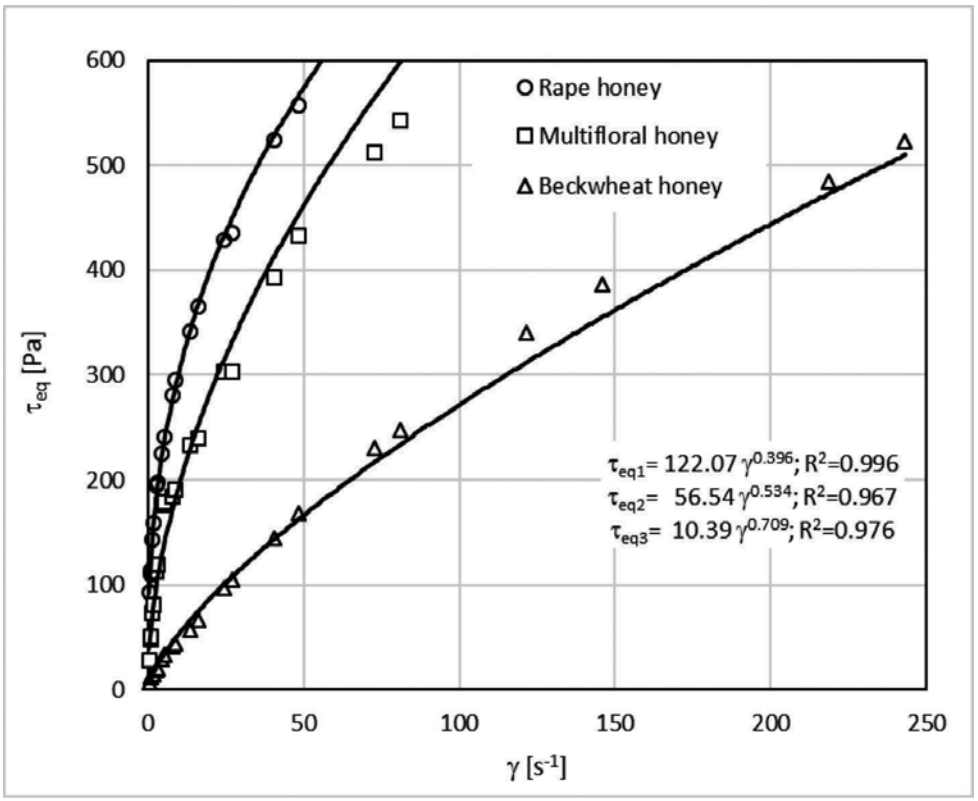

Figure 10. Equilibrium flow curves of media in crystallized state and at a temperature of T = 30°C [12].

$$\text{buckwheat honey} \quad \eta' = 10.39 \times \dot{\gamma}^{-0.291}. \tag{22}$$

Based on the data above, it can be stated that honeys with a fine-scaled structure are characterized by a higher value of apparent viscosity. This effect is even more noticeable in the form of a graph presenting the dependency of apparent viscosity in the function of mass fraction of the crystalline phase with low values of shear rate of $\dot{\gamma} = 0.5\ s^{-1}$ (**Figure 11**). It needs to be remembered that shear rate is a parameter which is strongly influencing the value of apparent viscosity.

Another characteristic effect presented by crystallized honey samples is its rheological instability. **Figure 12** shows characteristic hysteresis loops obtained in a shearing cycle with an increasing and then decreasing shear rate to a shear stress value of 500 Pa. The obtained hysteresis loops are characteristic for thixotropic fluids [19, 20]. All honeys in their crystallized state show a strong thixotropic effect, which can be measured using the hysteresis surface area. Nevertheless, it needs to be stressed that this effect is to a great extent permanent (the fluid does not fully rebuild its dormant-state properties) and is also connected with the destruction of the crystalline structure. During shearing, the breaking of small crystals occurs, which can be attributed to rheodestruction [20].

Crystallized honey samples show interesting behaviour in a dynamic rheological test. **Figure 13** shows the results of measurements of the same honey samples, which were rheologically characterized under rotational shearing conditions in **Figure 10**. The values of the viscosity mod-

ulus are a few times higher than of the elasticity modulus. As a result, the rheological properties of crystallized honeys are similar to those of viscous fluids. It is noticeable that the highest values of both the viscosity modulus and the storage modulus fall to the multifloral honey, while buckwheat honey is characterized by the lowest values. The values G' and G'' for rape honey are located between the values obtained for multifloral and buckwheat honeys, respectively. This behaviour shows that in relations to measurements conducted under rotary shearing conditions (**Figure 10**), there is both a quality and quantity change in the behaviour of the media.

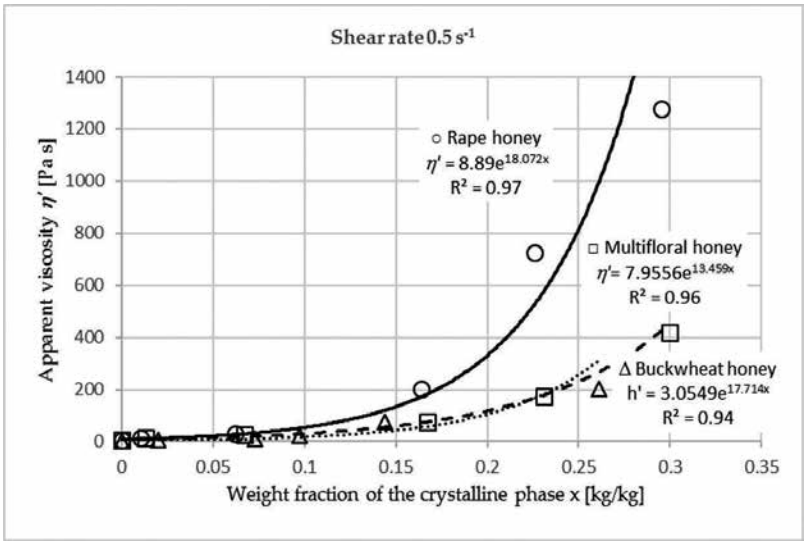

Figure 11. Dependency of apparent viscosity of crystallized honey on the mass fraction of crystallized phase for $\dot{\gamma} = 0.5\ s^{-1}$.

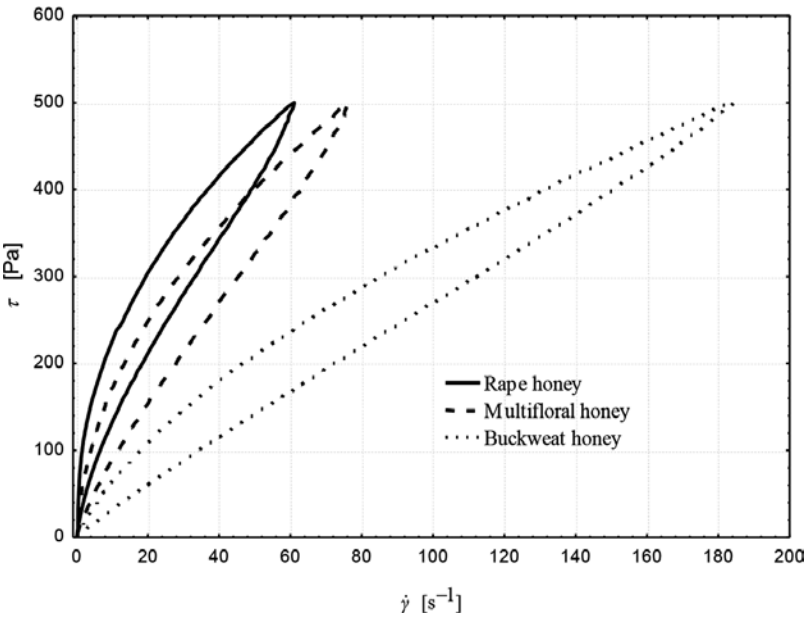

Figure 12. Characteristic hysteresis loops obtained for the analysed honeys for shearing with an increasing and then decreasing shear rate [12].

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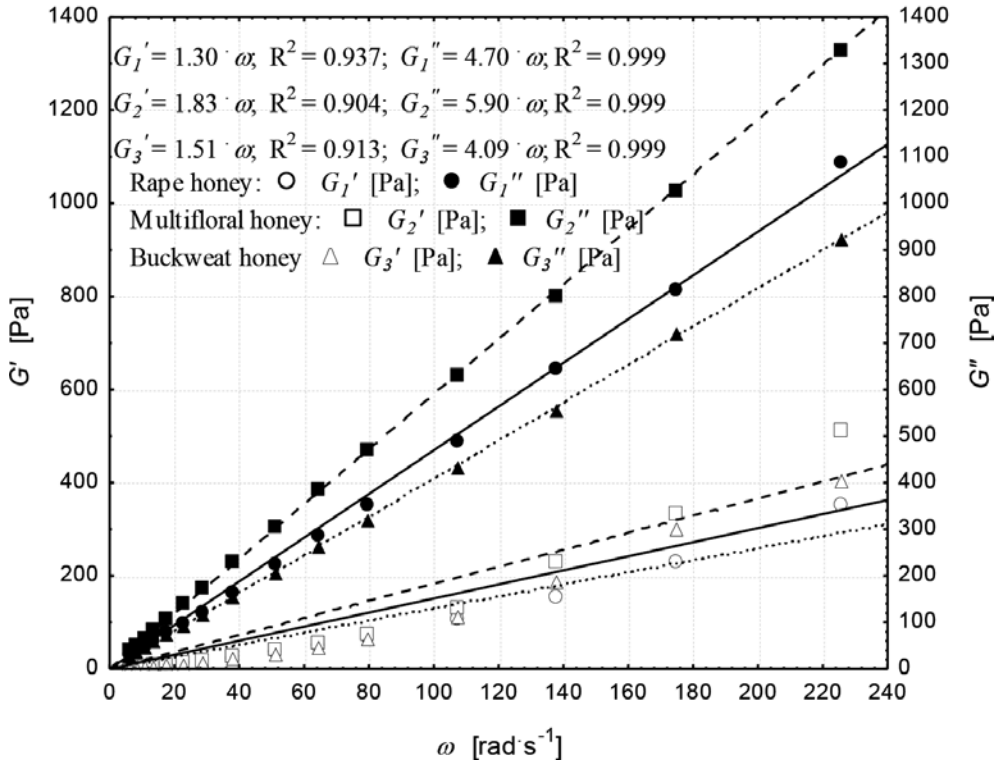

Figure 13. Values of the elasticity modulus and storage modulus of crystallized media in a function of angular oscillation frequency at a temperature of 30°C [12].

Differences in measurement results of rotational and oscillation measurements of crystallized honeys can be shown especially effectively by placing the values of apparent viscosity and dynamic viscosity on one graph. Such a graph is presented in **Figure 14**. Under oscillation shearing conditions, the highest values of complex viscosity were shown by crystallized multifloral honey samples, whereas under equilibrium shearing, the highest values of apparent viscosity were shown by rape honey samples (**Figure 10**). It needs to be stressed that both media were characterized by a similar water content and crystalline phase content. The parameter, which determined such behaviour, was mainly the morphology of the crystalline structure. The irregular shaping of crystals in multifloral honey samples under oscillation shearing (with constant shifts of the direction of deformation) generated higher movement resistance. It was thus noted that the manner of deformation of crystallized honey is a significant factor influencing the obtained rheological measurement results. Apparent viscosity of crystallized honeys decreases along with the increase of shear rate, whereas complex viscosity shows only slight changes with values close to constant.

Figure 14 clearly shows that crystallized honeys do not fulfil the Cox-Merz rule Coxa-Merza [20], since

$$\eta^* \neq \eta'\big|_{\omega=\dot{\gamma}}. \tag{23}$$

Nevertheless, there are such values of angular oscillation frequency and shear rate at which complex viscosity and apparent viscosity are equal to one another. These can be determined from **Figure 14**.

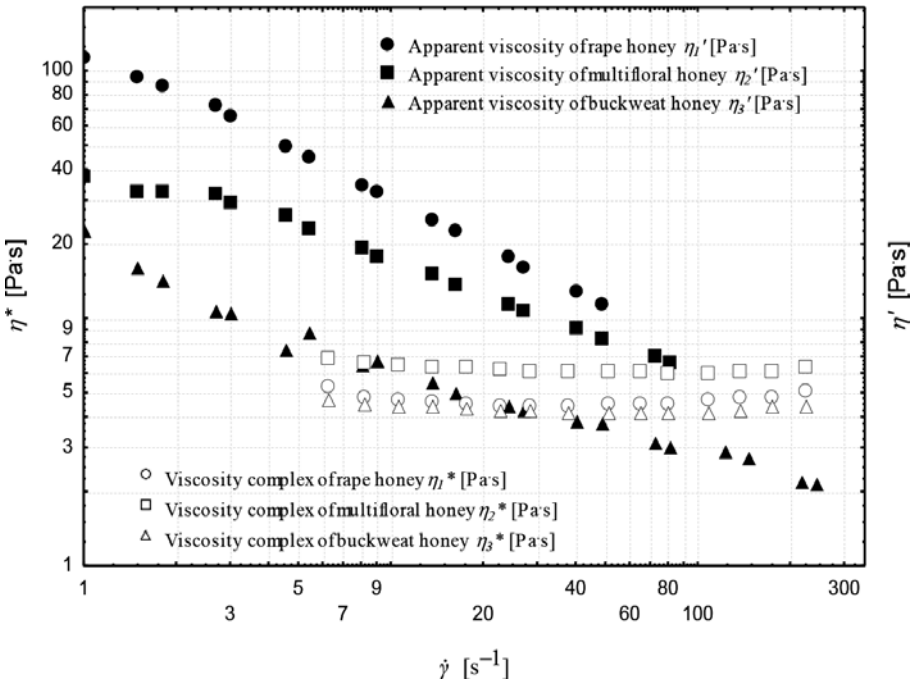

Figure 14. Presentation of the values of complex viscosity and apparent viscosity of crystallized honeys at a temperature of 30°C [12].

The results of rheological measurements of crystallized honey presented above do not exhaust the issue. The majority of the graphs shown in this text were obtained under specific conditions and it is hard to generalize them, as was the case with liquid honeys. Rheological studies of crystallized honey are extremely important in shaping the texture of the so-called creamed honeys. Creamed honey is obtained by the so-called direct crystallization with additional mixing during crystallization. This enables to deliberately shape the texture of crystallized honey to obtain characteristic features expected by consumers.

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Chapter 7

Fundamentals of Brazilian Honey Analysis: An Overview

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Andrei Felipe Moreira Buszinski,
Felipe Galeti Miguel, Gustavo Luis Cuba,
Thaila Fernanda dos Reis, Joelma Lambertucci,
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Additional information is available at the end of the chapter

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Abstract

Brazilian honey possesses large floral sources with various colors and flavors due to botanical and geographical differences and the large extension of the country. The absence of antibiotics and pesticides contamination positively differentiates Brazilian honey in the international market. Thus, the present chapter presents an overview of regulatory aspects for identity and quality evaluation of honey produced and commercialized in Brazil and international markets, as well as, it compares the production and consumption of honey with other countries. In addition, the chapter presents physicochemical and microbiological analysis commonly used in honey, as fundamentals of the technics and literature results with different kinds of honey obtained in Brazil. Physicochemical quality control and microbiological analysis of honey samples is of fundamental importance for assessing their quality, possible adulteration and storage conditions. In the literature, several methodologies exist to be used in the performance of honey quality control and each one complements the results in order to have an idea about the quality of the product,

the absence of adulteration, deterioration, and environmental pollution and geographical area. Finally, we will present the market scenario nowadays with future perspectives and some recognition obtained for Brazilian bee products in international events.

Keywords: Brazilian honey, regulation affairs, pollen analysis, physicochemical and microbiological analysis, contaminants, potential market, awards

1. Introduction

Honey is a natural product produced by bees from the nectar of flowers which can be modified by their digestive enzymes (floral honey) or from living plant fluids and/or excretions of plant-sucking insects (honeydew honey) [1].

Floral honey can be monofloral or polyfloral, depending on whether their production is derived from a single species or various species of plants, respectively. Polyfloral honey is universal, but monofloral honey can be produced by establishing hives where flowers of a particular plant species are dominant. Therefore, based on their peculiarity, unique flavors, and sometimes unique medicinal properties, monofloral honey has a higher commercial value. Manuka honey is an example of such type, which derives from two species of Leptospermum, and retail prices start at about $100/kg [2] due to its demonstrated health benefits [3]. In addition from the plant source, the commercial value and characteristics of the honey can also be based on insect source, as honey from stingless bees (e.g. Melipona beecheii) or honey from Apis mellifera, etc. exhibits different characteristics. Additionally, the absence of residues of contaminants may also play an important role in the international market, as in the case of Brazilian honey, which receives Organic Certification.

In this scenario, a variety of honey samples with different characteristics, biological effects, and commercial values are found worldwide. Because of the value of different types of honey could vary more than 100-fold, it is target for fraud. Reports have suggested the dilution of valued kinds of honey, such as from stingless bees, with low-value honey.

Biological honey activities are derived from compounds that are present in this natural food. In general, honey is composed of approximately 200 substances, particularly with those belonging to the classes of sugars, amino acids, proteins, organic acids, flavonoids, phenolic acids, vitamins, minerals, and volatile compounds. The chemical composition of honey is intrinsically related to factors such as the geographic region of origin, present flowers in this region, species of bee that produced it, climatic conditions, processing conditions, handling and storage, and the storage time [4]. Thus, honey chemical composition from different botanical areas can vary, also leading to differences to their biological properties.

Several efforts have been made worldwide to develop protocols aiming the identification and evaluation honey quality. The literature presents many methodologies that are used to determine honey identification and quality control, and they are complementary. Among them, it could be named ascertain the entomological sources of honey by pollen identification with checking of the morphological pollen of flowers present in each honey sample

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and quantification of the same [5] and physical and chemical tests, i.e. determination of 5-hydroxymethylfurfural (5-HMF), which aims to assess whether it has been stored properly and determine whether it is fresh, the determination of free acidity and pH, which can be used for checking the tampering and deterioration, respectively.

Additional or alternative methods to establish the plant source of an unknown honey have also been proposed through the genetic analysis of targeted gene regions isolated from honey. This technical approach was termed metabarcoding and it is gaining power because of increased access to high-throughput sequencing platforms [6].

According to the Technical Regulations for Honey identity and Quality of the Ministry of Agriculture, Livestock and Supply (MAPA) in Brazil [7], honey samples must be characterized by physical and chemical tests, such as moisture determination, minerals (ash), acidity, reducing sugars, apparent sucrose, insoluble solids in water, diastase activity, and hydroxymethyl furfural (HMF). These tests will be discussed deeply in the next sections, especially demonstrating the fundaments and importance of each one to guarantee honey quality in Brazil. Regarding the tests required worldwide, a comparison among the different regulations is also depicted. Additional assessments, which are not comprised in Brazilian Regulations, are also reported, as the determination of metals and pollen identification. Finally, honey market worldwide is exposed.

2. Regulation of honey in Brazil and in the world

The standard for honey was established in 1981 by CODEX Alimentarius organized by the World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) by delivering CODEX STAN 12-19811 to contribute to the safety, quality, and fairness of the international honey trade (see the parameters in **Table 1**) [8].

In general, each region of the world may also adopt regulations with parameters that will be committed to their market requirements, as well as, local environment. A summary of some important regulations with parameters and limits around the world is also demonstrated in **Table 1**.

Each parameter will be further explored in next section. In this section, however, it is important to demonstrate that Brazil is a country that has its own characteristics of climate, flora, and great biodiversity. This characteristic combined with the presence of Africanized bees allows the production of honey with its own characteristics of taste, purity, quality, and originality.

In Brazil, honey is a product regulated and supervised by the Ministry of Agriculture of Brazil, through the Federal Inspection Service (Serviço de Inspeção Federal—S.I.F) in accordance with Instruction No. 11, of October 20, 2000 [7, 9]. Because of the rustic characteristics of Africanized bees and richness of its flora, Brazilian honey has no residues of contaminants and is considered a high quality and pure honey which may be a product with Organic Certification.

Thus, one can observe that each country may establish its own quality parameters and there is still much to be aligned regarding the parameters and methodologies of analysis as they have many differences. With differences in parameters and methodologies, different results for the same honey sample may apply, leading to difficult negotiations between companies.

Parameter	CODEX Alimentarius [8]	Brazil (Instruction No. 11, of October 20, 2000) [7]	Europe (COUNCIL Directive 2001/110/EC of 20 December 2001) [10]	Japan [11]
Reducing sugars (fructose/glucose)	Not less than 60/100 g. Honeydew honey, blends of honeydew honey with blossom honey: not less than 45/100 g	Floral honey: minimum 65/100 g. Honeydew honey or mix of honeydew with floral honey: minimum 60/100 g	At least 60/100 g. Honeydew and honeydew mixes with nectar honey at least 45/100 g	Maximum 60/100 g
Moisture	Not more than 20%. Heather honey (*Calluna*)—not more than 23%	Maximum 20/100 g	Maximum 20/100 g	Maximum 20/100 g
Apparent sucrose	not more than 5 g/100 g. Alfalfa (*Medicago sativa*), *Citrus* spp., False *Acacia* (*Robinia pseudoacacia*), and others: not more than 10/100 g	floral honey: Maximum 6/100 g/ Honeydew or mix of honeydew with floral honey: maximum 15/100 g	Maximum 5/100 g. *Robinia pseudoacacia*, Medicago sativa, *Banksia menziesii*, *Hedysarum*, *Eucalyptus camaldulensis*, *Eucryphia lucida*, *Eucryphia milliganii*, *Citrus* spp.: Maximum 10/100 g *Lavandula* spp., *Borago officinalis*: Maximum 15/100 g	Maximum 5/100 g
Solid insoluble in water	Not more than 0.1/100 g Pressed honey —not more than 0.5/100 g	Maximum 0.1/100 g, except pressed honey, which is tolerated up to 0.5 g/100 g, only on products packaged for its direct sale to the public	Maximum 0.1/100 g	–
Electrical Conductivity	–	–	Maximum 0.8 mS/cm	Maximum 0.8 mS/cm
Minerals (ash):	Not mentioned	Maximum 0.6/100 g. Honeydew or mix of honeydew with floral honey is tolerated up to 1.2/100 g	Not declared	–
Pollen	Not mentioned	Honey must necessarily present pollen grain	Not declared	
Acidity	Not mentioned	Maximum of 50 mEq/kg	Maximum of 50 mEq/kg	Maximum of 50 mEq/kg

Parameter	CODEX Alimentarius [8]	Brazil (Instruction No. 11, of October 20, 2000) [7]	Europe (COUNCIL Directive 2001/110/EC of 20 December 2001) [10]	Japan [11]
Diastase activity	–	At least 8 on the scale of Göthe. Honey with a low-enzyme content should present at least 3 diastase activity on the scale of Göthe, where the content of hydroxymethylfurfural does not exceed 15 mg/kg	At least 8 on Schade scale. Honey with a low-enzyme content should present at least 3 on Schade scale, where the content of hydroxymethylfurfural does not exceed 15 mg/kg	–
Hydroxymethylfurfural	Not mentioned	Up to 60 mg/kg	Less than 40 mg/kg. But honey from tropical climate and blends of these honeys a maximum of 80 mg/kg is accepted	Maximum 50 mg/kg
Additives	Not mentioned	Absent	Not declared	Absent
Organic and inorganic contaminants	Honey shall be free from heavy metals in amounts which may represent a hazard to human health. The products covered by this Standard shall comply with those maximum levels for heavy metals established by the Codex Alimentarius Commission	Organic and inorganic contaminants and their limits are established by MERCOSUL Technical Regulation	Not declared	Dextrins: Absent Antibiotics: Absent High control regarding contaminants
Observations	The products covered by this standard shall comply with those maximum residue limits for honey established by the Codex Alimentarius Commission		In Germany, additional analysis of pyrrolizidine alkaloids (PA). It must be 50 μg/kg High control regarding genetically modified organisms—GMO	

Table 1. Parameters assessed in different regions of the world.

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3. Honey market worldwide and the potential market for Brazilian honey

In order to study the potential of Brazilian honey market, it is interesting to evaluate the production/demand of the honey around the world, the characteristic of the business, as well as, the conditions of Brazilian honey, production, and circumstances that can influence in this scenario. It is important to remember that the market is something very flighty and then, problems in one country, which presents high involvement in this business, can directly affect and change all circumstances and perspectives.

In this turn, it has been possible to observe a decrease in honey production in the US in last decades. Beehives in this country decreased from around 6 million in 1947 to 2740 million in 2016. This internal reduction in the production, from 250 million pounds in the early 1990s to approximately 178 million pounds in 2016, increased the demand for honey importation from other countries [12]. Colony collapse disorder (CCD) can be one of the reasons for the decrease in honeybee populations in the USA [13]. Despite the production reduction, honey consumption in the US has increased from approximately 400 million pounds in 2000 to approximately 450 million pounds yearly in last years. To maintain internal honey consumption, importation has increased from near 200 million pounds (in 2000) to 300 million pounds [14].

Argentina honey production was around 21 thousand tons in 1969, 110 thousand tons in 2005, and 80 thousand tons in 2013 [15]. This increase was mainly attributed to the clover plantation, since although the clover was planted to feed the cattle it gave a lot of nectar for honeybees to produce honey. Now it seems that honey production in Argentina was reduced due to the reduction of pasture and increase of soybeans, corn, and wheat plantations [16]. Only 8% of honey produced in Argentina is consumed in the internal market, making this country one of the biggest exporters of honey [17].

Europe produced about 372 thousand tons of honey in 2013, but it used to produce 309 thousand tons of honey in 1993. It is a great increase. But we have to understand better this market, as all of its self-production honey is consumed in its internal market. Europe is also a big importer country, importing about 305 thousand tons of honey in 2013, but it also exports a lot, in the same year they exported 176 thousand tons of honey [15]. That means that they still consume a lot of imported honey, but they re-export more than half of what they import with aggregated value. Europe is an important destination of Brazilian honey, especially because organic honey production is a very important point to be considered for these countries when importing honey, besides the absence of OGM pollen.

The China honey production increased from 75 thousand tons in 1969 to 450 thousand tons in 2013, and in this meantime the exportation in this year reached 125 thousand tons. Therefore, the internal consumption was around 325 thousand tons [15]. This data demonstrate non only the high honey production, but also, the high honey consumption for this country. China is the biggest honey exporter (in quantity) in the exporting universe, ahead of Argentina, which is also an important exporter. However, Chinese honey suffered an EU embargo in 2003, because of residue and antibiotic contamination found in honey.

On the other hand, after this Chinese honey embargo, Brazil has increased exportation. As previously mentioned, honey in Brazil is produced by Africanized bees, which are very strong

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bees, requiring no treatments with antibiotics or medicines. Therefore, Brazil presents the best bees for honey production [17]. In addition, Brazil has a great extension of territory, as well as rich flora and all resources to develop the bees [18] and honey production, without using antibiotics and pesticides, offering consequently a honey without contaminant residues.

Moreover, Brazilian honey production is mostly made in native areas, which also do not requires pesticides. Nevertheless, in 2006 Brazilian honey was banned from EU markets due to a lack of governmental Plan for Residues in honey [19], situation that was normalized in March 2008.

Brazil has the biggest extension of natural forest in the world and tropical weather in most of its area. That is about 300 million hectares of reserves, Indian territories and other protection areas for the biodiversity and rainforest, according to the IBGE. This allows Brazil to have the biggest organic honey potential production in the world [20]. Added to this huge area of natural forest, Brazil presented 6.9 million sq. km of eucalyptus planted area in 2014 [20]. In all areas of Brazil (north, south, southeast, northeast, and central west), eucalyptus can be cultivated. It is well known that eucalyptus plantation is very interesting for producing honey. It can be planted without chemical treatments allowing honey being produced as an organic area, producing also organic certified honey. Summing the area planted only for soybeans, coffee, cotton, orange, and sunflower in 2013 we can achieve an area of 100 thousand hectares (ha) as per IBGE [21]. Added to eucalyptus honey production and the other planted areas, it is important to consider that pollination services are rarely used in Brazil, and then, a large potential for increasing honey production could use this technique. Brazil has about 2.5 million bee colonies. Most of them are involved in honey production. Pollination is rarely used yet [22]. Brazilian honey productivity per hive is about 15 kg/colony/year. Comparing to Argentina with 35 kg/colony/hive [23], Australia with 118 kg/hive/year in average [24] and China 100 kg/hive/year, Brazilian beekeeping has much to grow [23]. It gives Brazil a possibility to increase honey production by using with techniques. Beekeeping in Brazil is very unprofessional. That is good, for one side, because no medicine, no antibiotic, and no special food is given to bees, maintaining the honey very natural. But productivity is low since it is very unprofessional yet. In the average, Brazil has a production of 30–40 thousand tons of honey yearly, since 2003 (**Figures 1** and **2**).

Brazilian exports have started in 2003 with China's honey embargo in the EU. Average honey exports are between 15 and 20 thousand tons yearly (**Figures 2** and **3**).

From the total honey produced, in 2014, 66% of it was exported. Brazil still has a very strong internal market for honey, however, with the price increase in last years because of intense exportation, internal Brazilian consumer is being suffering and then, the consumption can be reduced to a premium market only, i.e., consumers with a high-quality life.

Data have shown that honey consumption was 81 grams per capita in Brazil in 2014, an average really low comparing with other countries. Many programs are being conducted to distribute honey to governmental schools for the snack, but in the regular markets as drugstores and supermarkets consume is lower because of high pricing (**Figure 4**).

In conclusion, Brazil has the biggest potential to produce organic honey in approximately 100 square ha and approx. 7 square km of eucalyptus area. The Africanized honey bee, the best bee, is very resistant and using few techniques we can double per hive productivity. We have a potential internal market that can absorb honey production in the case of international

market unbalances. So, Brazil has the best potential to produce honey in the world with low risk. And can produce a very good and quality honey with organic certification.

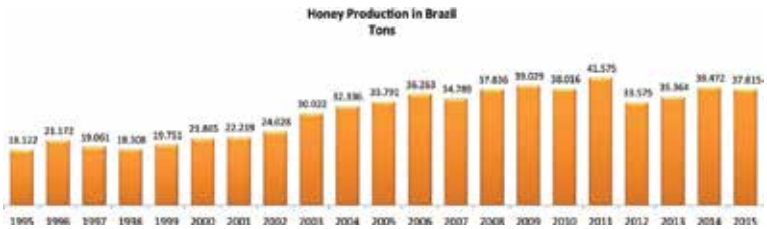

Figure 1. Brazilian honey production (tons). Data compiled for ABEMEL with information from: aliceweb.gov.br.

Figure 2. Brazilian honey production and exportation. Data compiled for ABEMEL with information from: aliceweb.gov.br.

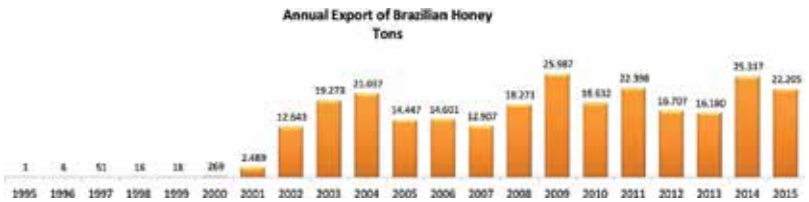

Figure 3. Brazilian honey exportation (tons). Data compiled for ABEMEL with information from: aliceweb.gov.br.

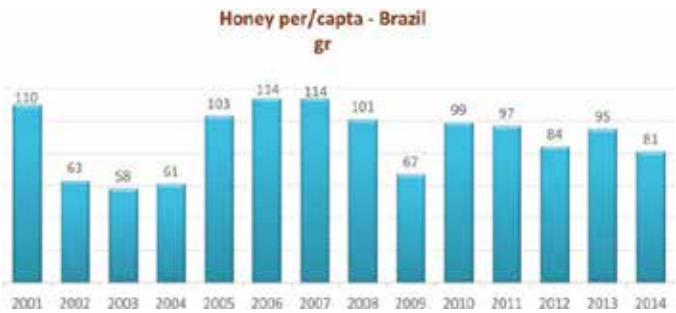

Figure 4. Brazilian honey per capita consumption (g)/year. Data compiled for ABEMEL with information from: aliceweb.gov.br.

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4. Pollen microscopical analysis

Microscopical analysis of pollen from bee products can offer several interesting information, as geographical source of the material (honey and propolis), botanical origin [25] and also can help about investigations involving contamination, yeast content (fermentation), dust, microscopic particles and others. In this last case, i.e. when the analysis is more complex and involve contamination investigation, this analysis is called palynological analysis [26].

Geographical origin and botanical source usually can be determined when pollen has not been completely removed by a technological process by filtration. Besides, in several countries pollen determination is not a requirement of quality; in Brazil, this point is requested by Normative Instruction no. 11, 2000 [7], and European Community is using a lot of morphological or DNA analysis in order to validate botanical or geographical source, besides OGM material (DNA analysis for this last one). Although this point is not a quality requirement for several countries, it can be used to confirm the geographical and botanical source, especially when some doubts appear. The pollen identification can be carried out using very simple and classical methods as microscopical morphological identification or using more advanced technologies as "DNA barcoding" [27]. The micromorphological analysis is very useful and the analysis can involve identification, as far as possible, of all pollen grains in the sediment, after properly preparation of the sample. The results can be expressed as an (i) estimate value, (ii) determination of frequency classes, and the (iii) count expressed in percentage. For the first case, it is necessary to count around 100 grains and elements correspondent, in the second, around 200–300 pollen grains, in this case, if the pollen is of only a few species, around 200 pollen grains is enough, and finally, in the last case, the presentation of the frequencies as percentage is possible counting around 1200 pollen grains, with two slides counted [25].

When the honey is classified according to plant source, the common name or botanical name is written with word "honey" (CODEX STAN 12-1981) [8]. The MAPA use classical methods as the reference and the results are compared with the literature. The São Paulo's state government has a databank with more than 17,000 slides, but the access it is only in loco (http://botanica.sp.gov.br/palinologia/palinologia-colecao-cientifica-palinoteca/). Nevertheless, there is electronics databank available, as picture bank of Universidade de São Paulo (http://www.lea.esalq.usp.br/polen/) [28].

The pollen analysis also used to classify the honey as monofloral or unifloral, when the dominance of pollen of a single plant species, the bifloral dominance of pollen of only two plant species and plurifloral or heterofloral with no dominance of pollen of any plant species. Dominant pollen occurs in honey sediments above 45%, at least 300 pollen grains counted. This kind of classification is commercially important because monofloral honey is the most valued since it keeps the same physicochemical and organoleptic characteristics [26].

Despite the facility of preparation of slides in the traditional method, the interpretations of results and time involved with pollen grain counting sometimes is a challenge, in this way molecular tools could be applied. The "DNA barcoding" could be used to identify source plants in the honey. In this method, a short sequence of the DNA of the standardized portions

of the genome is used and the results are compared with a reference database, as the GenBank [27]. DNA markers, such as nuclear 18S rDNA, the plastid trnL gene, plasmid coding regions rbcL and matK, trnH-psbA and ITS2, were used to test their ability to identify plant traces from different honey samples, and [27] suggested that the rbcL region and the trnH-psbA spacer could be considered to establish the origin, quality, and safety of honey with DNA barcoding, since besides more studies are necessary the stakeholder was established. In order to exemplify the microscopically analysis of pollen in Brazilian honey samples, our group evaluated five samples, including two samples of orange honey, one sample of plurifloral honey, one of "cipó-uva" honey, and a sample identified by beekeepers as "coffee" honey, that in fact is a plurifloral one, since only a very few amount of coffee pollen was found in the sample. **Figure 5** shows some pollen identified in the honey samples evaluated, and **Figure 6** shows the microscopical image of the pollen obtained in two increases 20 and 40×, usual way to count pollen grain on honey samples (for sample preparation, see [25]).

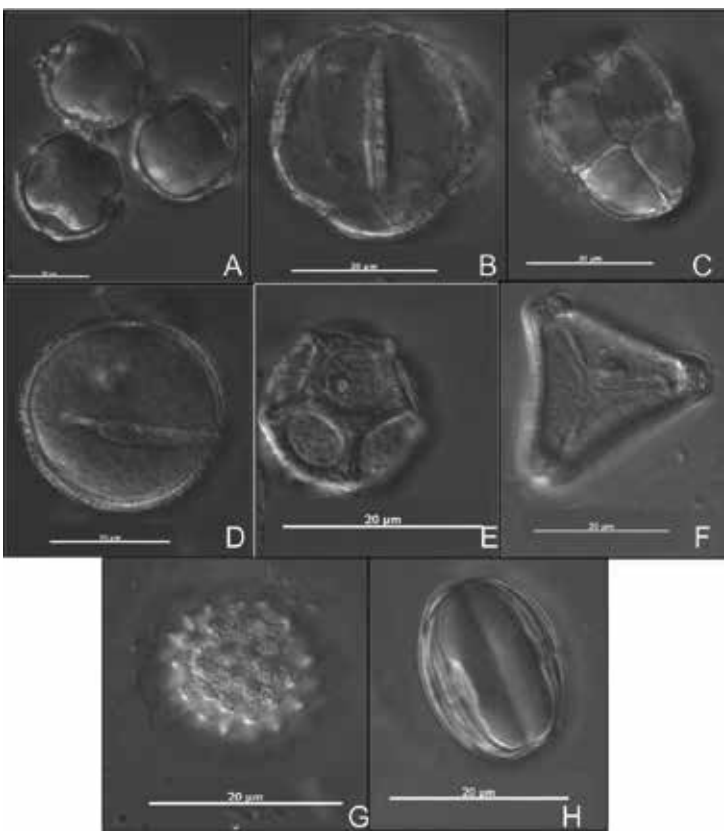

Figure 5. (A, B) Orange pollen, *Citrus* sp. (Rutaceae); (C) *Mimosa* sp. (Mimosaceae); (D) Coffee pollen, *Coffea arabica* (Rubiaceae); (E) *Alternanthera* sp. (Amaranthaceae); (F) "Cipó-uva" pollen, *Serjania* sp. (Sapindaceae); (G) "Vassourinha-do-campo pollen," *Baccharis* sp. (Asteraceae), and (H) Melastomataceae. All slides were viewed with a Carl Zeiss (Jena, Germany) microscope using the 100× magnification oil immersion objective. Phase contrast brightfield was taken with an AxioCam camera (Carl Zeiss). Images were processed using the AxioVision software version 3.1 and saved as TIFF files. Photographs were taken by Nathália U. Ferreira and Thaila F. dos Reis.

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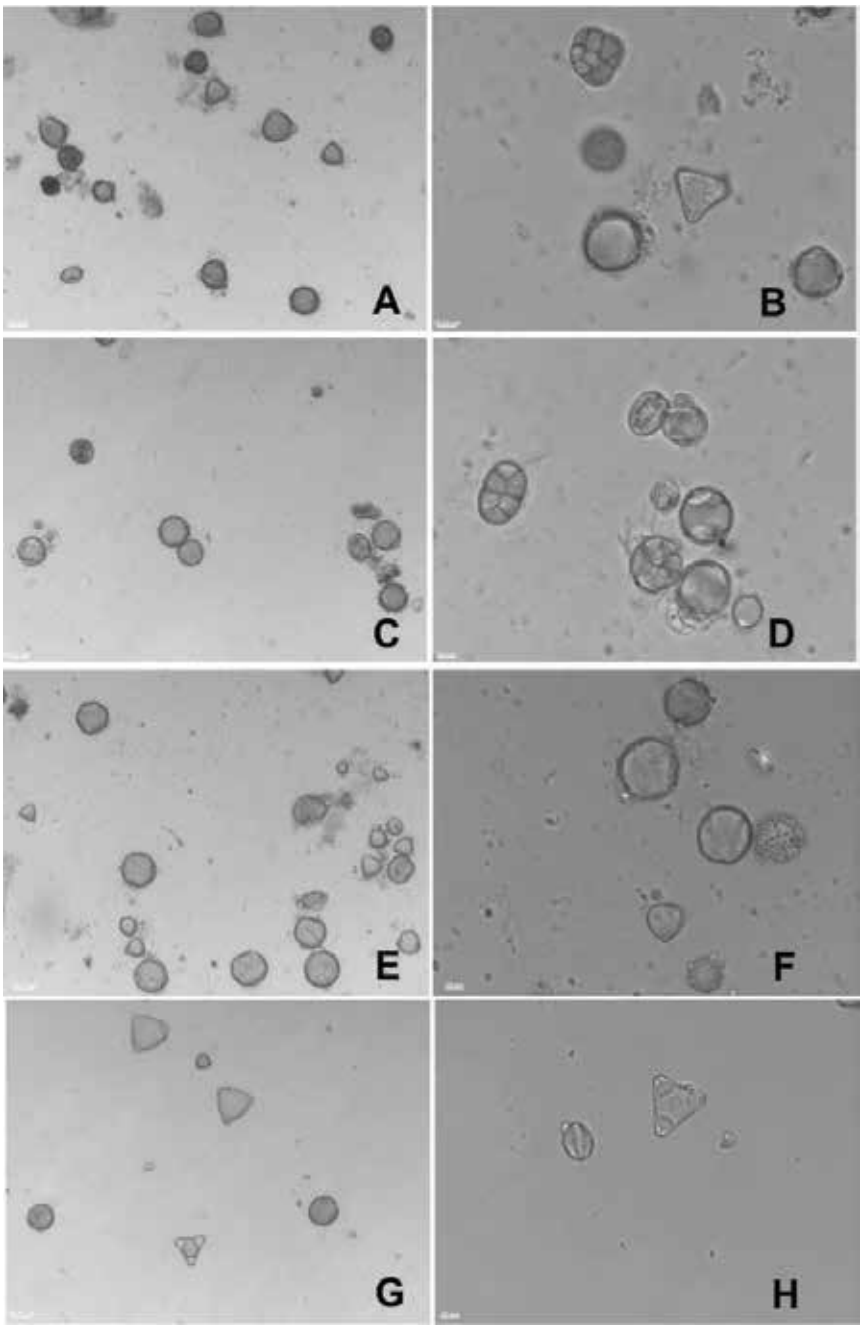

Figure 6. Microscopical analysis of honey samples obtained from different geographic and botanical areas. (A, B) Plurifloral honey, (C, D) orange (*Citrus* spp.) honey, (E, F) coffee (*Coffea arabica*) honey, and (G, H) "Cipó-uva" honey (*Serjania* spp.) (20 and 40× increase, respectively). All slides were viewed with a Carl Zeiss (Jena, Germany) microscope using the 40× magnification oil immersion objective or 20× lens. Phase contrast bright field was taken with an AxioCam camera (Carl Zeiss). Images were processed using the AxioVision software version 3.1 and saved as TIFF files. Photographs were taken by Thaila F. dos Reis.

5. Physicochemical analysis of honey: fundamentals and objectives of the analysis and results for some Brazilian samples

The honey chemical composition is intrinsically related to factors as bee species, geographical origin, flora, climate conditions, seasons, processing, manipulation, and storage conditions [4]. Brazil that presents a large biodiversity is able to offer several different types of honey, as shown in **Figure 7**, bees visiting "pau-Brasil" flowers and "cipó-uva" honey in the comb. As mentioned previously, in general, honey consists of approximately 200 substances including sugars, amino acids, proteins, organic acids, flavonoids, phenolic acids, volatile compounds, vitamins, minerals, pigments, wax, enzymes, pollen grains, and other phytochemicals [4, 29].

Figure 7. (A) Honeybees collecting nectar from "Pau-Brasil" (*Caesalpinia echinata*) flowers, showing the high biodiversity and infinite possibilities of floral honeys. (B) "Cipó-uva" (*Serjania* spp.) honey in honeycomb. Photography (A) was taken and gently donated by Mr. Antônio Carlos Meda, and photograph (B) was taken and gently donated by Lucas Eduardo Meda, both from Apis Flora Indl. Coml. Ltda, Ribeirão Preto, São Paulo, Brazil.

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Quality control analyses are extremely important in the evaluation of origin, quality, adulteration, storage conditions, and contamination of honey. The physicochemical properties of a honey sample may provide important information about its biological and geographic origin [30]. Honey adulteration, mainly due to the addition of sugar derived from sugar cane, corn, and beet or even by providing sugar as a food source for bees, occurs due to its limited availability and high cost [31]. Suitable storage conditions are essential to ensure honey quality, as its chemical composition may change due to the thermal process, oxidation, and fermentation reactions [4]. Nowadays, the increasing use of pesticides in agriculture makes contamination of honey by its residues a public health issue [32].

Taking it into consideration, analytical methods are essential to provide reliable results. In the literature, there are several methodologies employed in honey quality control analyses, which are complementary for honey samples appreciation. In 1990, the International Honey Commission (IHC) was created with the goal to generate a new world honey standard. All employed honey analyses methods were then collaboratively tested and published as "Harmonised Methods of the European Honey Commission" [33]. Based on this fact, the EU Honey Directive and Codex Alimentarius Standards were revised for honey analyses. Since then, IHC continuously aims to improve and develop new analytical methods for honey analysis.

5.1. Color

The color of honey is an important quality parameter for commercialization as it is its first attractive attribute [4]. The color is directly related to its chemical composition, ash content, temperature of the hive, and it changes during storage time [34]. The main compounds related to the color of honey are phenolic compounds, pollen and mineral contents, which may vary widely according to its botanical and geographical origin [30]. During storage, the color of honey may change due to the fermentation process such as caramelization and Maillard reactions or due to the thermal process, which may change its chemical composition and consequently its color [35] or according the package used. To determine the color of honey, a photometer with direct readout in mm Pfund may be used. The Pfund scale compares an analytical standard scale of reference on the graduation of glycerin in order to provide repeatable and accurate results [30]. According to the Codex Alimentarius Committee on Sugars [8], color of honey may vary from nearly colorless to dark brown.

Regarding Brazilian honey color analysis, Sodré et al. [36] studying 36 honey samples from north coast of Bahia found predominance of the light amber color (75%) followed by amber color (16.6%) and in minor proportion, dark amber, extra light amber and extra white colors (with 2.8% each one). Moreti et al. [37] analyzed 52 samples of honey from several counties of Ceará state and found colors as water white (26.92%), white and extra white (17.31% each one), light amber (15.38%), extra light amber (11.54%), amber (9.61%), and dark amber (1.92%). **Figure 8** presents the different colors observed in only five samples studied here showing how different honey could be from Brazil especially because of the large biodiversity and extension of the country.

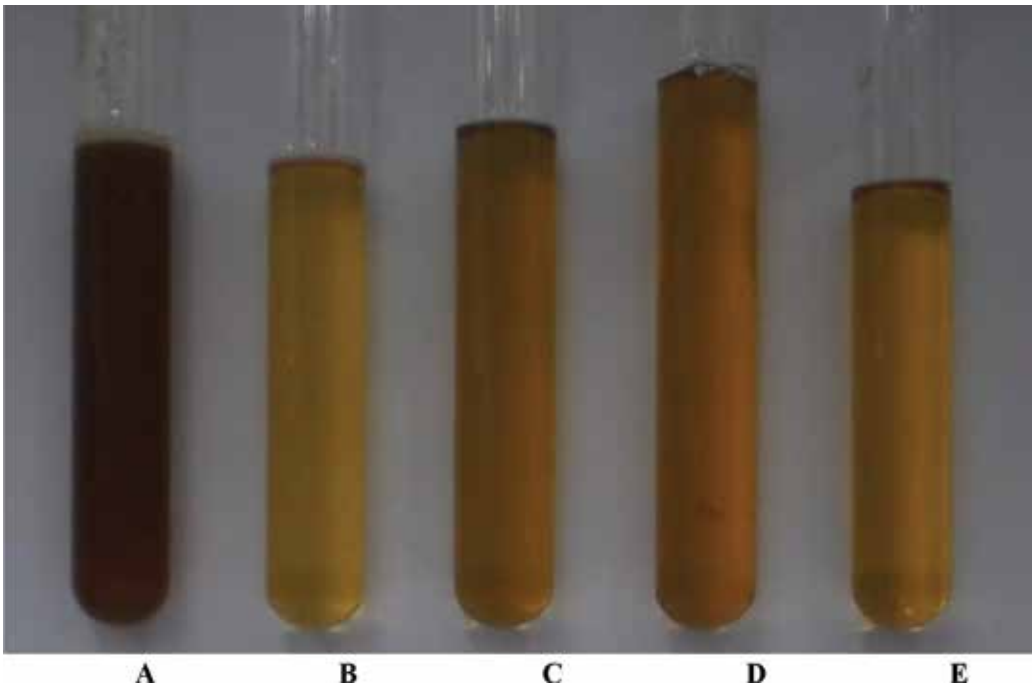

Figure 8. Color and botanical source according beekeepers about the samples used in this work, respectively, from left to right: Polifloral honey (Apiary Joel Souza, Altinópolis/São Paulo—batch 019400916) and orange (*Citrus sinensis*) honey (Apiary Hugo Charnet, Galvão Peixoto/São Paulo—batch 019300815), coffee (*Coffea Arabica*) honey (Apiary Roberto Quintino, Minas Gerais) samples gently donated by Apis Flora Indl. Coml. Ltda, Ribeirão Preto/São Paulo, Brazil. The second orange (*Citrus sinensis*) honey sample (Baldoni, batch 1607) followed by "Cipó-uva" (*Serjania* spp.) honey (Baldoni, batch 1484) were produced in Baldoni, Campinas/SP, Brazil and were acquired in Santa Terezinha Empório, Ribeirão Preto/São Paulo.

5.2. Moisture

The water content in honey samples varies according to botanical origin, climate conditions, processing techniques, and storage conditions [4]. Moisture influences honey's properties such as viscosity, crystallization, solubilization, color, and flavor [38]. The moisture may increase during processing and storage time and should be evaluated since its increase makes honey more susceptible to the fermentation process [39]. Determination of moisture in honey samples can be performed employing a refractometric method, which is based on the increases of refractive index related to solid content, and so it is possible to determine indirectly moisture of honey. According to the Codex Alimentarius Committee on Sugar [8], the moisture content in honey should not exceed 20% [8].

Several authors described the moisture content found in Brazilian honey samples. Périco et al. [40] analyzed 30 samples from Toledo, Paraná and found values ranging from 8.7 ± 0.3 to 17.6 ± 6.8/100 g. In the Rio Grande do Norte, Soares et al. [41] analyzed 24 samples from 12 commercial points of Apodi, RN, and found higher values of moisture, ranging from 16.5 to 21.5/100 g. In turn, Paulino et al. [42] found similar values of moisture (15.2–20.33/100 g) when analyzed 13 samples from various cities of Ceará state. Some examples of moisture and parameters described are shown in **Tables 2** and **3**.

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Parameter	Rates	Local (state)	Reference
Color	Extra white to dark amber	Bahia Ceará	[36, 37]
Moisture (g/100 g)	15.2–20.33 16.5–21.5 8.7 ± 0.3–17.6 ± 6.8	Ceará Rio Grande do Norte Paraná	[40, 41, 42]
Ash content (%)	0.3 ± 0.10 0.17–0.20 0.01–0.41 0–1.34	Bahia Paraíba Ceará Ceará	[36, 42, 44, 46]
Electrical conductivity (μS/cm)	780.7 ± 302.70 192.00–798.67 179–198 120–750	Bahia Ceará Ceará Ceará	[36, 46] [45] [42]
pH	3.77 ± 0.25 2.90–5.10; 2.30–5.00; 2.70–4.60 3.53–4.60	Bahia São Paulo Paraná	[36] [49] [40]
Free acidity (mEq/kg)	29.10 ± 7.04 12.50–55.00; 14.00–75.50; 14.00–57.00 26.73–126.77	Bahia São Paulo Rio Grande do Norte	[36, 41, 49]
Sugars (%)	*Reducing sugars* / *Apparent sucrose* 69.20 ± 1.82 / 2.40 ± 1.42 78.84 ± 2.71 / 2.71 ± 2.40 62.89–86.93 / 1.13–10.12	Bahia Ceará Rio Grande do Norte	[36, 41, 46]
5-HMF (mg/kg)	20.70 and 23.90 7.00–355.50 70.62–150.27 31.28 ± 0.2–581.4 ± 4.2	Paraíba Ceará Rio Grande do Norte Paraná	[40, 41, 42, 44]
Diastase (Gothe scale)	34.11 ± 8,41 5.30–43.39 1.10–38.50	Bahia Ceará São Paulo	[36, 46, 49]

Table 2. Presentation of results obtained with different geographic and floral honey found in Brazil.

Honey Physicochemical Parameters	Polifloral*	Orange*	Coffee*	Orange#	"Cipó-uva"#
Aspect	Homogeneous, viscous liquid	Homogeneous, viscous liquid	Homogeneous, viscous liquid	Homogeneous, viscous liquid	Homogeneous, viscous liquid
Color	Dark amber	White, very clear yellow	Yellow, clear	White, very clear yellow	White, very clear yellow
Density (g/mL)	1.415 ± 0.00	1.420 ± 0.00	1.425 ± 0.00	1.426 ± 0.00	1.435 ± 0.00
Moisture (% w/w)	15.90 ± 0.00	15.80 ± 0.00	16.00 ± 0.00	16.10 ± 0.00	14.70 ± 0.00
Total ash (%w/w)	0.03 ± 0.03	0.03 ± 0.03	0.07 ± 0.03	0.08 ± 0.03	0.05 ± 0.01
pH determination	3.76 ± 0.01	3.78 ± 0.01	3.60 ± 0.01	3.51 ± 0.01	3.73 ± 0.01
Free acidity (%w/w)	0.19 ± 0.00	0.15 ± 0.00	0.19 ± 0.00	0.16 ± 0.00	0.18 ± 0.00
HMF determination** (mg/Kg)	55.2 ± 0.60	12.1 ± 0.20	16.5 ± 0.20	28.3 ± 0.10	32.2 ± 1.40

Honey Physicochemical Parameters	Polifloral*	Orange*	Coffee*	Orange#	"Cipó-uva"#
Insoluble material (%w/w)	0.05 ± 0.02	0.00 ± 0.00	0.02 ± 0.01	0.00 ± 0.00	0.01 ± 0.01
Reducing sugars (%w/w)	71.1 ± 0.60	70.2 ± 0.60	69.1 ± 0.60	70.9 ± 0.70	77.0 ± 1.80
Apparent sucrose (%w/w)	3.47 ± 0.43	2.80 ± 0.02	2.76 ± 0.02	3.48 ± 0.03	4.12 ± 0.37

*Gently donated by Apis Flora Company and #Baldoni Company.
**HMF was determined using spectrophotometry UV methodology.

Table 3. Physical-chemical analysis of different floral sources of Brazilian honeys (n = 3).

5.3. Ash content and electrical conductivity

Ash content and electrical conductivity are parameters mainly used to measure mineral content, which may be an indicative of environment pollution, the geographic and botanical origin of the honey [4, 39]. Mineral content is also associated with sensorial properties as color and flavor, which are important for honey commercialization [38]. Ash content provides important information about the quality of honey, as floral honey has lower ash content than honeydew honey [30]. Determination of ash content is performed by a gravimetric method [43]. The Codex Alimentarius Committee on Sugars [8] does not recommend a specific value for ash content. Electrical conductivity is related to the presence of ions, organic acids, and proteins in honey [4]. The determination of this parameter is based on the measure of the electrical resistance, which is reciprocal of the electrical conductivity [43]. According to the Codex Alimentarius Committee on Sugars [8], it is recommended a maximum value of 800 mS/cm for the electrical conductivity of honey samples.

Paulino et al. [42] found ash content in Brazilian honey ranging from 0 to 1.34%. According to Brazilian legislation, the ash content in blossom honey should be at maximum 0.6%, and at maximum 1.2% for honeydew honey [7]. Rodrigues-Evangelista et al. [44] found values from 0.17 to 0.20% of ash when analyzed honey samples from Paraíba state. Sodré et al. [36], in turn, found an average of 0.3 ± 0.10% of ash content in honey from Bahia state. In another study, the same group found values ranging from 0.01 to 0.41% of the total ash.

Bendini and Souza [45] analyzed 24 samples of blossom honey derived from cashew flowers from Ceará state and found electrical conductivity values from 179 a 198 μS/cm with an average of 187 ± 4.8 μS/cm. When 13 honey samples from Ceará state were analyzed by Paulino et al. [43], values ranging from 120 to 750 μS/cm were found. Sodré et al. [36] found an average of 780.7 ± 302.70 in 36 samples of bee honey from Bahia state and when honey samples from Ceará were analyzed by the same group, values between 192.00 and 798.67 μS/cm[1] were found [46].

5.4. pH and free acidity

The presence of organic acids in honey is responsible for its natural acid pH value. Determination of pH in honey samples is important to confirm its authenticity, as an addition

of sugar in honey significantly increases pH values [47]. Free acidity is characterized by the presence of organic acids in equilibrium with their respective lactones, esters, and inorganic ions [29, 48]. It is a parameter used to evaluate honey deterioration, as fermentation of sugar into organic acids increases its value [30]. The determination of free acidity in honey is performed by a potentiometric titration method and the results are expressed in milliequivalents of acid per kg of honey [43]. The Codex Alimentarius Committee on Sugars [8] recommends a maximum value of 50 mEq/kg for free acidity in honey.

When 30 samples of honey from Paraná state were analyzed, Périco et al. [40] found pH values ranging from 3.53 to 4.60. Soares et al. [41] determined the acidity value in 24 bee honey samples from Apodi, Rio Grande do Norte and found results ranging from 26.73 to 126.77 mEq/kg. In turn, Sodré et al. [36] determined the pH and acidity value in 20 bee honey samples from Ceará state and their average were 3.77 ± 0.25 and 29.10 ± 7.04 mEq/kg, respectively. Marchini et al. [49] also analyzed the same parameters in 205 honey samples from different localities from São Paulo state and found pH values of 2.90–5.10 to eucalyptus honey, 2.30–5.00 to wild honey, 2.70–4.60 to orange honey, and acidity values of 12.5–55 mEq/kg of eucalyptus honey, 14–75.5 mEq/kg to wild honey, and 14–57 to orange honey.

5.5. Sugars

Sugars are intrinsically related to the flowers used by bees to produce honey, climate, and geographical conditions. Monosaccharides are the most common sugar in honey and fructose (38.5%) and glucose (31.0%) are the major sugars in honey [47]. The ratio of fructose and glucose in honey samples are used to evaluate the degree of crystallization of the honey sample [50]. Determinations of reducing sugars and apparent sucrose are based on a titrimetric method employing Fehling's reagent. The method is a titration of a Fehling's solution at boiling point by reducing sugars in honey using as indicator methylene blue [43]. Determination of the ratio of fructose and glucose may be performed by quantification of sugars in honey samples by GC methodology employing a sugar derivatization process or by HPLC methodology employing a refractive index detector or a pulsed amperometric detection [43, 51, 52]. The Codex Alimentarius on Sugars [8] stipulates that the minimum content of reducing sugars in floral honey is 60 /100 g.

In 2003, Sodré et al. [36] found an average of 69.20 ± 1.82% of reducing sugars and 2.40 ± 1.42% of apparent sucrose. The same group analyzed in 2006, 20 samples from different regions of Ceará state and found 78.84 ± 2.71% of reducing sugars and 2.71 ± 2.40% of apparent sucrose. Soares et al. [41] found a reducing sugar content of 62.89–86.93% and apparent sucrose from 1.13 to 10.12% in 24 samples of 12 providers from Apodi, the Rio Grande do Norte.

5.6. 5-HMF

Sugars present in honey may alter during storage time due to nonenzymatic reactions such as Maillard reaction, caramelization, and sugar degradation [47]. The compound 5-hydroxymethylfurfural (5-HMF) is a decomposition product of monosaccharides present in honey. Factors such as temperature, heating, floral origin, pH, and storage conditions may significantly influence in 5-HMF content [53]. Therefore, 5-HMF content is a parameter used to

determine the freshness of honey, as it is absent in fresh honey and its concentration increases during storage time [30]. Furthermore, high 5-HMF content may indicate adulteration of honey by the addition of invert syrup [47]. Determination of 5-HMF content may be performed employing a spectrophotometric method [43], or a chromatographic method by HPLC using calibration curves of 5-HMF analytical standards to quantify this compound in honey [43, 53]. The Codex Alimentarius Committee on Sugars [8] stipulates 5-HMF content at the maximum value of 40.00 mg/kg and, if honey is from a tropical region, accepts a maximum value of 80.00 mg/kg.

The HMF values found in Brazilian honey are higher than those found in nontropical countries, as Paulino et al. [42] that encountered 7.00–355.50 mg/kg in 13 samples from several cities of Ceará. Périco et al. [40] also found high HMF values (31.28 ± 0.2 to 581.4 ± 4.2 mg/kg) when analyzed 30 samples from Toledo, Paraná, and Soares et al. [41] found values ranging from 70.62 to 150.27 mg/kg. When honey from two distinct regions (São João do Cariri and Areia, both in Paraíba state) were analyzed by Rodrigues-Evangelista et al. [44], the HMF content was between 20.70 and 23.90 mg/kg.

5.7. Diastase

Diastases are enzymes present in honey, which are sensitive to heat and consequently, may be used to evaluate honey overheating [47]. Therefore, the measure of diastase activity is an indicative of honey's freshness and is useful to detect improper storage conditions [30]. Diastase activity may be also an indicative of honeybees fed artificially with glucose, as a diastase enzyme deficiency is observed in this case [54]. The determination of diastase activity is based on a spectrophotometric kinetic method, which measures the activity of diastasis enzymes present in honey, in order to monitor adulteration by the addition of sugar and evaluate storage time and conditions [4, 43]. For that, under specific conditions, the activity of diastase enzymes of honey is measured in a standard solution of starch. The Gothe unit is used to express diastase activity and is defined as the amount of enzyme which will convert 0.01 g of starch in 1 hour at 40°C [43]. The Codex Alimentarius Committee on Sugars [8] stipulates a minimum value of 8.00 Gothe; however, a minimum value of 3.00 Gothe is accepted for honey with low diastase activity if the 5-HMF content is lower than 15 mg/kg.

The diastase activity was determined in 20 samples of honey from Ceará state by Sodré et al. [36] and found an average of 34.11 ± 8.41 (in Gothe scale). Sodré et al. [36] analyzed 36 honey samples from Bahia and found the value between 5.30 and 43.39. Marchini et al. [49] analyzed 205 honey samples from different localities of São Paulo state and found values ranging from 1.10 to 38.50, with an average of 8.14 for orange honey, 15.77 for eucalyptus honey, and 17.32 for wild honey. For different floral sources, the authors found values ranging from 7.80 to 19.00.

In complement to pollen microscopical analysis, physical-chemical results for these Brazilian honey samples were conducted and which is presented below, where it is possible to demonstrate the identity and quality of some floral sources of Brazilian samples studied here.

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6. Microbiological analysis of honey: fundamentals and objectives of the analysis and results for Brazilian samples

Currently, safe food is a major global public health concern, since food may be contaminated by pathogenic microorganisms, which can cause severe diarrhea or debilitating infections. Furthermore, microorganisms can be responsible for the spoilage of food. Besides the contamination by microorganisms, food may be contaminated by chemical substances, such as toxins, environmental pollutants, and heavy metals [55].

Honey has low susceptibility to the proliferation of microorganisms due to its physicochemical characteristics, such as antimicrobial substances, low moisture content (low water activity), low pH, and oxidation reduction potential, among others [56]. Therefore, its antimicrobial properties discourage the growth or persistence of many microorganisms. Nevertheless, honey may be contaminated by primary and secondary sources of microbial contamination. Primary sources, including pollen, nectar, digestive tracts of bees, dust, air, and soil, are difficult to control. Secondary sources of contamination (after-harvest) include cross-contamination, equipment, food handlers, among others, and may be controlled by good manufacturing practices. Regarding the harvesting method, honey samples harvested using modern methods (colony established in man-made bees' accommodation called hives) have lower yeast and bacterial counts than samples harvested using traditional methods (honey hunting, which use flame to destroy the insects and are used in honey bee colony established in wood logs), that is, modern methods are more hygienic and produce the better quality of honey. Furthermore, exposure of colony to fire also kills bees and hampers the process of cross-pollination and may lead to consumption of the whole forest [57].

The honey samples should be subjected to quality control tests to evaluate their physicochemical and microbiological parameters. Thus, it is possible to assess whether the results are within specifications and detect if there was an adulteration of honey. In Brazil, the Ministry of Agriculture, Livestock, and Supply (MAPA) published the Technical Regulation of Identity and Quality of Honey (Brazil, 2000), which describes that the analysis of contaminants should follow the Technical Regulation of the Southern Common Market (MERCOSUL or MERCOSUR). Regarding microbiological criteria, the document "MERCOSUL/GMC/RES n° 15/94" has the following technical specifications for honey: total coliforms/g: absence; *Salmonella* spp. and *Shigella* spp./25 g: absence; enumeration of molds and yeasts: maximum of 100 CFU/g [58].

According to MAPA, microbiological methods recommended by the International Organization for Standardization (ISO) should be used [59]. The enumeration of coliforms is performed using the colony-count technique (ISO 4832:2006) [60]. The total coliform group includes four genera: *Escherichia*, *Klebsiella*, *Citrobacter*, and *Enterobacter*. The presence of these bacteria in food indicates that there was fecal contamination. Therefore, they are commonly used as indicators of sanitary quality of honey. Some authors evaluated Brazilian honey samples from different regions of Brazil (states of Ceará, Bahia, Pernambuco, Piauí, Rio Grande

do Norte, and the Rio Grande do Sul) and showed that there was absence of coliforms in these samples [9, 61–63], that is, the harvesting, the management, and the processing of the samples were made as recommended in order to obtain a good quality of honey.

The detection of *Salmonella* spp. should be performed using the International Standard ISO 6579:2002 [64]. The genus *Salmonella* includes several pathogenic serotypes, which can cause from gastroenteritis (fever, diarrhea, and abdominal cramps) to serious systemic infections (enteric fevers), like *Salmonella* typhi that causes typhoid fever. However, gastroenteritis is the most common form of salmonellosis and the major mode of transmission is by means of contaminated food. Some studies showed that there was an absence of *Salmonella* species in Brazilian honey samples from different regions of Brazil [9, 61, 63, 65].

The enumeration of yeasts and molds, in its turn, is performed using the colony-count technique according to ISO 21527-2:2008, which specifies a method for the enumeration of viable xerophilic molds and osmophilic yeasts in products that have a water activity less than or equal to 0.95 [66]. Luiz et al. [65] evaluated Brazilian honey samples produced in several cities of the state of Minas Gerais (Southeast region), and the yeast and mold counts varied from <10.0 to 3.3×10^1 CFU/g, that is, all samples were according to Brazilian law. In another study by Schlabitz et al. [63] with honey samples from state of Rio Grande do Sul (South region of Brazil), the majority of samples (10 samples) were within specifications, since the enumeration of yeasts and molds varied from $<1.0 \times 10^1$ to 8.0×10^1 CFU/g. However, two samples had values above 100 CFU/g:1.3×10^2 and 6.1×10^2 CFU/g, respectively. Several honey samples produced in the state of Ceará (Northeast region of Brazil) were evaluated by Santos and Oliveira [61]. The authors showed that the majority of samples were within specifications, since yeast and mold counts varied from < 10.0 to 6.0×10^1 CFU/g. Only one sample had a count above 100 CFU/g, since it had 1.8×10^2 CFU/g.

Although not required by Brazilian law, the detection of *Clostridium* spp. (spore-forming bacteria) also is important, since honey samples may be contaminated with spores of *Clostridium botulinum*, the etiological agent of botulism (potentially fatal disease). While the ingestion of these spores is considered harmless to healthy adults, the spores may germinate in the gut of infants under 6 months of age, multiply and produce botulinum toxins. This would not occur in children older than about 6 months and adults due to natural defenses that develop over time [55]. Ragazani et al. [68] evaluated honey samples from different regions of Brazil (states of São Paulo, Minas Gerais, Goiás, Ceará, Mato Grosso, and Santa Catarina) and isolated *C. botulinum* from 7% of the samples. In other studies, Schlabitz et al. [63] and Luiz et al. [65] showed that there was an absence of sulfite-reducing clostridia in Brazilian honey samples from states of Rio Grande do Sul and Minas Gerais, respectively.

7. Contaminants analysis: metals, pesticides, analysis, and results

Honey is traditionally consumed by humans for being considered a product of natural origin and healthy. However, honey and other bee products can also be a source of toxic substances,

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such as antibiotics, pesticides (insecticides, fungicides, herbicides, and bactericides), heavy metals, bacteria, and radioactive materials due to environmental pollution and misuse of beekeeping practices, for example, when these substances overdose in beehive treatments. Honey bees collect pollen and nectar from the flowers and then they may return to hives collecting significant amounts of toxic contaminants, therefore their hives and products can result contaminated with many different kinds of pollutants [69, 70]. Thus, the monitoring of contaminants in honey is necessary to warrant consumers' safety.

7.1. Pesticides and antibiotics

The presence of contaminants in bee products decreases its quality and it may carry serious health hazards, consequently, being a public health problem. Widely used in agricultural practices, pesticide residues have been shown to cause genetic mutations and cellular degradation and the presence of antibiotics might increase resistant human or animal's pathogen [71].

The pesticide residues may originate from the treatment of beehives with acaricides and organophosphorus pesticides (OPPs) in the control of Varroa jacobsoni and Ascosphaera apis. Indirect honey contamination can occur during pesticide application in agriculture also for wax moth and small hive beetle control. Pesticide application in crops can contaminate soil, air, water, and the flowers from which bees collect nectar for honey production [72, 73].

Another source of contamination are the antibiotics such as tetracyclines, streptomycin, sulfonamides, and chloramphenicol used for the treatment of bee disease, migration from wax to honey, and also of some infestations such as Varroa destructor, Acarapis wood, and Paenibacillus larvae [69, 72].

The determination of pesticide in food due to the low concentration, the distinct chemical properties, and the matrices complexity, requires sample preparation, purification, identification, and quantification of compounds. Therefore, honey is a complex matrix and this implies the need for effective clean-up treatment before the analysis. Among the extraction methods commonly used in honey analysis are the typical clean-up/extraction procedures, such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE); however, they have the disadvantages of being expensive and using large amounts of organic solvents, which are generally toxic for the technician and can contaminate the environment and usually enable the extraction of analytes belonging to only one chemical class [32, 70]. Additionally, there are other extraction techniques, which have been employed to reduce a number of reagents and time spends on sample preparation, for example, supercritical fluid extraction (SFE), matrix solid phase dispersion (MSPD), solid phase microextraction (SPME), and stir bar sorptive extraction (SBSE). Besides the extraction and purification procedures, the choice of the separation/detection approach is of fundamental importance. The step of identification and quantification of pesticide residues in honey is based mainly on gas chromatography (GC) or high-performance liquid chromatography (HPLC) techniques, both coupled with tandem

mass spectrometric detection have shown great success in the multiresidue analysis of antibiotics and pesticides in honey [71].

Rissato et al. [74] confirmed 48 pesticides of different classes (organohalogen, organophosphorus, organonitrogen, and pyrethroids) in low levels in Brazilian honey samples (Bauru, São Paulo, Brazil) by gas chromatography-mass spectrometry (GC-MS/MS). Nevertheless, malathion residues were detected in all the samples, in a high concentration, and it was attributed to pesticide application for dengue vector control in the area. A study realized by De Pinho et al. [73] showed that of the 11 honey samples from eight regions of the state of Minas Gerais (Brazil) analyzed only two presented chlorpyrifos and k-cyhalothrin residues using liquid-liquid extraction with low-temperature purification for pesticide residue analysis by gas chromatography. However, the concentrations obtained were below the maximum residue levels (MRLs) established for pesticides in foods products. The presence of these compounds was confirmed by mass spectrometry (GC-MS).

Additionally, Orso et al. [75] developed and validated a method for the simultaneous determination of 79 pesticides and 13 antibiotics for 43 honey samples from different regions of Rio Grande do Sul State, Brazil, among them are monofloral and multifloral honey. The pesticides and antibiotic residues were extracted using a water-acetonitrile followed by a cleanup with dispersive solid phase (d-SPE) and analyzed by UHPLC-MS/MS. The results of the analysis demonstrated that 50% of the samples presented residues of one or more analytes in the samples. The maximum residue limit was not exceeded in any sample. Residues of insecticides and acaricides, fungicides, antimicrobials, and herbicide were found at concentrations below the MRLs, according to the limits established by National Program for Honey Residues Control established by the Brazilian Ministry of Agriculture (Brazil) for honey. Second, the authors, the residues found in honey samples are due to the proximity of the beehives with soybean, corn, or wheat crops, considering that bee realizes the pollination process, reaching large distances to collect nectar, water, and pollen of flowers.

7.2. Heavy metals

The bees are exposed to metals contained in pollen or nectar, it can to accumulate them and finally into the honey produced from it [76]. A number of different minerals and heavy metals in honey are largely dependent on the soil composition, as well as various types of floral plants [77]. Additionally, metal pollutants are discharged into the air, water, and soil through mining, agriculture practice, waste dump, coal burning, hydraulic fracturing to extract gas and oil, and industrial and municipal waste production. Agroecosystems fertilized with manures and biosolids can become contaminated with metals, and repeated fungicide application can cause the buildup of metals [78].

Trace metals such as sodium, potassium, calcium, iron, zinc, and copper can be considered essential for the biological metabolism of living organisms, when present in optimum concentrations are helpful. Other metals such as lead, cadmium, mercury, and aluminum are classified as microcontaminants of the environment, toxic or nonessential to living organisms,

and at high concentrations can be even lethal, due to the inability of the heavy metal to be metabolized by the body, leading to accumulation in human or animal soft tissues without being fully inactivated or destroyed [77, 79]. In addition, the problems caused by heavy metals include headaches, metabolic abnormalities, respiratory disorders, nausea, vomiting, damage to the brain, kidney, nervous system, and red blood cells [77].

The methods used to determine the chemical elements in honey are based on spectroscopy or spectrometry techniques (including flame emission photometry or spectrometry (FES), inductively coupled plasma optical emission spectrometry (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS), flame atomic absorption spectrometry (FAAS), electrothermal atomic absorption spectrometry (ET-AAS), graphite furnace atomic absorption spectrometry (GF-AAS), hydride generation-atomic fluorescence spectrometry (HG-AAS), ion chromatography EDTA titration) [77].

De Andrade et al. [80] determined the trace elements, Pb, Cd, and Cr in 52 honey samples from eight different regions from the state of Paraná (Brazil), using slurry sampling and graphite furnace electrothermal atomic absorption spectrometry. The mean concentration of the elements followed the order Pb > Cr > Cd, but the study concluded that honey samples from Paraná have food security, as regular consumption of this product does not put risks to human health in terms of intake of this metallic species. Furthermore, Batista et al. [81] determined 42 chemical elements (toxic and essential elements) in Brazilian honey samples collected in different cities of Brazil (poli, orange, and sugarcane flowers) by the inductively coupled plasma mass spectrometry method. The authors observed that in general Brazilian honey presented higher mean concentrations for Ni, Mg, and Al and lower mean concentrations of Pb, Cd, and Cu. The mean values found for P, Zn, Mn, and Fe were very similar to those found in honey samples from other countries.

Thus, the presence of pesticides and antibiotic residues and trace metals in honey is of interest for quality control and also as a bioindicator of environmental contamination. Therefore, these analyses are important to determine the nutritional value and also the potential effect of honey on human well-being, and they can be called upon to ensure the general safety and purity of honey.

8. Brazilian honeybee products recognized around the world

Besides the quality observed in physicochemical and microbiological parameters, the absence of pesticides, antibiotics, and residues in general, it is important to recognize that several Brazilian honeybee products were awarded in important fairs and competitions around the world. The awards varied since color, taste, and flavor until high technology involved. Considering honey, several awards were attributed to Brazilian Companies (**Figure 9**). A Company situated in the State of Santa Catarina was awarded during several Apimondia Conferences. The dark honey received Gold Medal in Australia in 2007. The varieties of Dark honey were also awarded Gold Medal in Ukraine in 2013, followed by

a Bronze Medal for clear honey. In 2015, this same Brazilian company was awarded one gold medal, two silver medals, and one bronze medal, for two varieties of honey and two creamy kinds of honey, during Apimondia that was held in South Korea. Another company focused on bee derivative products situated at Campinas, in the State of São Paulo. The gourmet honey of this company was awarded for superior taste in ITQI, International Taste and Quality Institute from Brussels in 2016, besides this important recognition, the

Figure 9. Photographs presenting some important awards for honey and Propolis from Brazil, as a demonstration of the international recognition of the quality and (A) Prodapys' representative, Mr. Célio Hercilio Marcos da Silva and Mr. Tarciano Santos da Silva, receiving four awards obtained for different types of Brazilian Honey in Apimondia 2015, Ukraine. (B) Baldoni's representative, Mr. Gustavo Delfino Calomeni and Mr. Daniel Augusto Cavalcante, receiving Gourmet's Honey award in Conbrapi conference that was held in Fortaleza, 2016. (C) Natucentro's representative, Mr. Cezar Ramos Júnior, receiving award for best photography of Green Propolis being produced by bees, Apimondia 2016, Ukraine. (D) Essenciale's owner, Nivia Alcici, receiving award for Gourmet Red Propolis wine, SIAL Innovation China, 2015; and finally (E) Dra. Andresa A. Berretta, from Apis Flora Indl. Coml. Ltda, receiving the second place for the development of a mucoadhesive gel containing propolis, Royal Academy of Engineering, Leaders in fellowship, London, 2015. All photographs were gently donated by the owners.

better taste was again attributed to this company in CONBRAPI, a Brazilian Conference, during the years 2012, 2014, and 2016, in Gramado/Rio Grande do Sul (RS), Belém/Pará (PA), and Fortaleza/Cerá (CE), respectively. These several awards can demonstrate the differences in taste, color, and flavor from Brazilian different geographical and botanical sources.

Brazilian propolis is also a very important honeybee product from Brazil, very recognized internationally, not only with several biological properties described but also considering the chemical differences of this type of propolis in comparison with the others found around the world, especially because of prenyl derivatives of p-coumaric acids, such as Artepellin C. Some companies received awards for propolis products as an award for Technological Innovation at China SIAL Fair in 2015, with two Gourmet line products, "Duo propolis green and red wine extract" and "premium red propolis extract wine." Another two medals were received with better photographs of bees collecting and producing green propolis, during Apimondia 2013 (Ukraine) and Apimondia 2015 (South Korea). And finally, a Brazilian Company situated at Ribeirão Preto, São Paulo state was selected for Royal Academy of Engineering Innovation Training because of the development of a mucoadhesive gel with propolis to treat vulvovaginal candidiasis. During the selection of the better project and presentation, Andresa A. Berretta was awarded the second place.

9. Conclusion

In conclusion, it is possible to show that some little differences in quality parameters exist between different countries/regulations because it is related to the floral sources. Several techniques are now available for the most of the analysis required and the most recent methodologies usually are more sensible than the oldest. Several different types of honey can be found in Brazil because of large extension of the country and the important biodiversity of each region. These differences directly affect the physical-chemical quality and also the presence contaminants. In general, it is possible to show that Brazilian beekeepers can improve techniques to increase honey production and Brazilian honey is very well recognized around the world especially because of the absence of residues, pesticides, and heavy metals, offering an Organic Certified honey and with very especial and nice taste.

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Chapter 8

Physicochemical Characterization of Maltese Honey

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Additional information is available at the end of the chapter

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Abstract

The Maltese Islands are renowned for the production of genuine honey from different floral sources depending on the season and the location of the apiary. Honey samples were collected directly from local beekeepers over a period of 4 years. Each sample was coded and the details provided by the beekeepers were recorded. A total of 259 samples were collected. The distribution of the apiaries was also considered for the three honey seasons: spring, summer and autumn. All samples were tested for the parameters according to the EU Directive on Honey (2001/110/EC) and the Harmonised Methods of the International Honey Commission (2009). The samples were analysed for consistency (by appearance), moisture content and Brix (by refractometry), colour index, diastase, proline and hydroxymethylfurfural (by spectrophotometry), pH and electrical conductivity (by pH/conductivity meters), salinity (chloride meter), free acidity (by titrimetry), polyphenols (by the Folin-Ciocalteu test), sugar content (high performance liquid chromatography), antioxidant activity (by DPPH and FRAP) and antimicrobial activity. The Maltese honey can be classified into three seasons with distinctive physicochemical characteristics. Honey originating from particular season showed significantly different values for specific parameters. Typically, high sucrose content is found in spring honey and a high conductivity in autumn honey.

Keywords: Maltese honey, physicochemical, seasons, sugars, polyphenols

1. Introduction

1.1. Maltese Islands and local honey history

The production of high-quality Maltese honey has been renowned since ancient times. The Ancient Greeks and Romans used to call the island ελίτη (Melite) meaning "honey-sweet". Under the Arab rule, the name “Melite” was changed to “Malta”.

1.2. Historical aspect

In ancient history, honey from Malta was considered as a delicacy and was also exported from the island. Research suggests that it was the Phoenicians who introduced the domestication of beekeeping in Malta. The Phoenicians brought the knowledge of apiaries and earthenware jars construction. In fact some Punic apiaries cut out in the rock still remain today (**Figure 1**). During the Roman rule beekeeping continued in the Maltese Islands, as observed by the presence of Roman beehives. Honey was very important to the Roman Empire and documents were discovered where people who stole honey were brought to justice. The Maltese honey was renowned for its spicy and blended taste [1].

In the Maltese Islands, there are a number of sites and places that have names originating from the apicultural industry e.g. "Wied il-Għasel" which means Valley of Honey and "Imġiebaħ" which means Apiaries. These names from the apicultural industry and the many ancient remains found around the island prove the basis of the bee population and honey production on the Maltese Islands.

The production of honey in ancient times was much less than today's production. In fact, there was only one harvest season, the wild thyme honey season, which was on the 26th of July (religious Feast of St. Anne) [1]. The traditional techniques used in bee-honey production started to change in the 1950s as the first movable frame hives and tools were introduced. The hives and tools needed were generally imported from Britain. The hives imported were copied and then produced locally. Till today, the British Standard hive is usually used in Malta. The biggest drop in the use of jars was in the early 1990s when a Varroa mite infestation resulted in the elimination of about two-thirds of the entire bee colonies on the islands [2].

1.3. The Maltese bee

The Maltese Islands have their own endemic bee type, called *Apis melliferaruttneri* [3]. This endemic sub-species of honeybee is known to have inhabited the Maltese Islands for

Figure 1. An ancient apiary in Malta, dating from the Punic era. The jars used to be placed in the wall holes. The beekeepers used to enter from the small door to add extensions to the back of the jars.

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centuries. It is a sub-species of the Western honeybee or European honeybee (*Apis mellifera*), but is different from other Mediterranean bees. Up to recent times, it was the sole honeybee species in the Maltese Islands.

The Maltese bee is slightly smaller in size, dark in colour with apparently no yellow bands, and is resistant to certain diseases, but is vicious and highly active. Comparing the Maltese bee to the North African bee and the Sicilian bee, it results that it has shorter legs and wings. The wings of the Maltese bee are also much narrower. It has also a very wide abdomen. The hair on its abdomen is also very long. After centuries of local colonization, the Maltese bee has adapted well to the Maltese Islands climate and environment [3].

However, *Apis melliferaruttneri* is a very productive bee as it can work on windy days and also during very hot weather. During the hot summer of Malta, when temperatures can increase to 40°C, the bee tends to work early in the morning and in the afternoon till sunset while taking a short break during mid-day. From local beekeepers' observations, it cleans the hive very well and removes any foreign material promptly. Nevertheless the drawback is its aggressiveness.

1.4. Local honey production

The local honey is produced from different floral sources depending on the season and the location of the apiaries. In Malta, beekeepers usually harvest three times during the year; the first harvest takes place in spring, the second harvest in summer and the third harvest in autumn.

The first type of honey that is harvested in the Maltese Islands is the spring multi-flora honey. This type of honey is produced from the nectar of several types of flowers that are present in spring time. Typical plants include red clovers (*Hedysarum coronarium*), bore thistle (*Galactites tomentosa*) and starflower (*Borago officinalis*). This type of honey is collected during May and has the tendency to solidify in a few months [1].

The second type is the summer wild thyme honey. Wild thyme (*Thymus capitatus*) is a shrub that starts flowering by late May in Malta. The nectar of wild thyme produces honey which has a very delicious and spicy taste. In the summer season, honey usually starts in the last week of May and ends between the end of June and the first week of July. This honey has been very famous since ancient times and is a type of honey that is sought by both locals and foreigners.

The last season of Maltese honey production is the autumn season. This honey season usually starts from the month of August and ends in November. By the end of August the bees start collecting nectar from the flowers of Eucalyptus (*Eucalyptus melliodora*) and later in October, nectar from the carob trees (*Ceratonia siliqua*). Some beekeepers harvest the eucalyptus honey and then afterwards harvest the carob honey. Other beekeepers harvest only once in late autumn to produce a multi-floral honey made from eucalyptus flowers, carob flowers and some other flowers that the bees might find in the beginning of the season. Local tradition states that honey produced from carob is good for sore throats and for people who smoke.

The historical unique attributes that have been assigned to the Maltese honey must be due to the vast range of wild flowers within the Maltese Islands. There are about 1000 species of

mostly wild flowers identified in Malta such as wild thyme, white clover, bitumen clover, white mustard and borage [4]. The carob, citrus and stone fruit trees also help to give the honey a more special taste.

1.5. Aims of the study

This research was primarily characterised by two main aims:

(a) To determine the quality of Maltese honey. The main attributes were studied in accordance with the Food Safety Act Honey Regulations [5], the Council Directive 2001/110/EC [6] and the Harmonised Methods of the International Honey Commission [7]. Thus, local honey was valued by colour designation, sugar content, moisture content, water insolubility content, electrical conductivity, free acid, diastase activity, pollen types and HMF (hydroxymethylfurfural) content. Additional analyses included antioxidant activity (by DPPH and FRAP) and antimicrobial activity.

(b) To determine any particular characteristics for honey collected from the three honey seasons. Geographical map of the foraging areas around Malta and Gozo was one of the outputs.

2. Materials and methods

2.1. Honey samples

Honey samples were collected from Malta, Gozo and Comino between 2011 and 2014. All samples were obtained directly from the beekeepers. Each honey sample was assigned a code that was used throughout the experimental trail. Information on the physical status (colour, consistency, etc.) of the honey samples, and location data were recorded for future reference. The seasonal distribution on a yearly basis is illustrated in **Table 1**. No winter samples were collected as the foraging area usually lacks plants in flower during this season. Beekeepers were provided with their honey analysis for their information.

2.2. Physical appearance: colour and consistency

The colour of the honey samples was determined by the absorbance measurement at 560 nm using the UV-Vis spectrophotometer (WPA Lightwave II). Values exceeding 2.5 A were

	Spring	Summer	Autumn
2011	21	34	22
2012	42	34	12
2013	9	5	10
2014	34	27	9
	106	100	53

Table 1. The collection of honey samples by season and by year.

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diluted and then the absorbance value was multiplied by the dilution factor to obtain the final absorbance. The viscosity of the honey sample was determined on the physical appearance of the honey.

2.3. Determination of brix and moisture content in honey

Before any measurements the refractometer was calibrated with distilled water. Approximately 0.3 ml of sample was placed on the prism. For each honey sample, three consecutive readings with independent honey were recorded for brix and moisture content.

2.4. Determination of pH and free acidity of honey

A honey sample of 10 g was accurately weighed in a 250 ml beaker on an electronic balance. The honey sample was then dissolved in 75 ml of carbon dioxide-free water (freshly produced de-ionised water) in a 250 ml beaker. The pH was recorded (Orion Star A215 Thermo Scientific). For the measurement of free acidity, the above solution was titrated with 0.1 M NaOH solution to pH 8.30 using the automatic titrator (Kern. Model: ABT-120-5DM).

2.5. Determination of electrical conductivity

A solution containing 20% of honey dry matter in 100 ml distilled water [7] was prepared for each sample. The 20% dry matter was determined from the moisture content reading. The amount of honey, equivalent to 20.0 g anhydrous honey, was dissolved in 70 ml distilled water, and made to a 100 ml volume. The conductivity was determined using a pH/conductivity meter (Orion Star A215 Thermo Scientific) in μS/cm) in triplicates.

2.6. The determination of HMF after White

The method according to White [8] was followed. Briefly, 5 g of honey were weighed accurately and dissolved in 25 ml of distilled water. 0.5 ml of Carrez solution I and 0.5 ml of Carrez solution II were added, and the solution made to a volume of 50 ml with water. Following filtration and dilution, the absorbance of the samples was read at 284 and 336 nm in 10 mm quartz cells within 1 hour. The HMF content in mg/kg was then obtained.

2.7. The determination of diastase activity

The Megazyme test kit (Megazyme Ireland, lot number 30602) was used for this determination. Briefly, 2 g of honey sample was dissolved in 40 ml of 100 mM sodium maleate buffer (pH 5.6) and topped to 50 ml. The Amylazyme tablet was added and following an incubation period of 10 min at 40°C, 10 ml of Trizma base (2% w/v) solution were added. The absorbance of the solution was read at 590 nm. The α-amylase activity of a sample (as Schade per gram of honey) was determined by use of the associated regression equation.

2.8. The determination of proline

The method outlined in Ref. [7] was followed. Briefly, 5 g of honey were made to the 100 ml volume with distilled water. 0.5 ml of the sample solution in one tube, 0.5 ml of water (blank test)

into a second tube and 0.5 ml of proline standard solution into a third tube were pipetted. 1 ml of formic acid and 1 ml of ninhydrin solution were added to each tube. After 15 min shaking, the tubes were incubated at 70°C for 10 min. 5ml of the 2-propanol-water-solution were added to each tube and the absorbance read at 510 nm after 45 min. The proline content in mg/kg honey was calculated.

2.9. Determination of sugar content

The respective standards and honey samples were prepared as 1% solution prior to analysis. Each standard and sample (2 µl) was injected in triplicates in a Dionex Thermo Fisher Ultra High Performance Liquid Chromatography with a charged aerosol detector, equipped with an amino column from Supelco (250 × 4.6 mm, 5 µm particles). Mobile phase consisted of a water/acetonitrile mixture (volume ratio 75/25), with a flow rate of 1.5 mL/min. Detection was performed at a Data Collection Rate of 20 Hz, filtered at 5 s, peak width was 0.02 mm and oven temperature set to 35°C.

2.10. Polyphenolic content

The total phenolic content (TP) was determined using a Folin Ciocalteu test [9]. 100 µl of Folin-Ciocalteu reagent and 80 µl of sodium carbonate (1 M) were added to 10 µl of each honey stock solution (triplicates) and incubated for 20 min at room temperature. The absorbance was read at 630 nm. Tannic acid was used as a standard for the test.

2.11. Total flavonoid content

The total flavonoid (TF) content was determined by a spectrophotometric method [10, 11]. Honey samples were prepared as 50% (w/v) solutions. 25 µl of honey solutions and 100 µl of 0.15% $NaNO_2$ solution (Fisher Scientific, UK) were allowed to mix for 6 min. 100 µl of 4% NaOH solution and 25 µl of distilled water were added for a final volume of 250 µl. After 15 min, the absorbance was read at 510 nm. Rutin (Sigma-Aldrich, USA) was used as a standard for the quantification of the total flavonoid content (0–500 mg/mL; r^2= 0.9962) [12].

2.12. Radical scavenging activity: the DPPH assay

The DPPH assay (2,2-diphenyl-1-picrylhydrazyl, Sigma Aldrich, USA) was carried out according to Moien et al. [10]. Honey samples were prepared as 12.5% (w/v) solutions. 200 µl of a 100 µM solution of DPPH radical in methanol were added to 20 µl of honey solution, and incubated for 30 min in the dark at room temperature. The radical inhibition was measured at 490 nm against a blank containing DPPH and methanol. Ascorbic acid (BDH, UK) was used as a standard (10–100 µg/mL). The AAE-DPPH and ascorbic acid equivalence (mg AEAC/100 g honey) (r^2= 0.9928) were calculated [12].

2.13. Reducing power: the FRAP assay

The FRAP (Ferric Reducing Antioxidant Power) activity of honey samples was determined according to Oyaizu [13]. 250 µl of honey solutions (6.25–50%) or 250 µl of distilled water

(blank) were mixed with 250 μl of 0.2 M phosphate buffer (pH 6.6) and 250 μl of 1% potassium ferricyanide. Following an incubation period of 20 min at 50°C, mixtures were immediately cooled in an ice bath for 30 s. 250 μl of 10% trichloroacetic acid were added and centrifuged at 3000 rpm for 10 min. 500 μl of the upper layer were mixed with 500 μl of distilled water and 100 μl of 0.1% ferric chloride. The absorbance was read at 700 nm against a blank [14]. The EC_{50} was used to define the specific reducing capability (mg AEAC/100 g honey) using ascorbic acid (10–100 μg/ml; r^2= 0.9981) as a positive control.

2.14. Antimicrobial activity

Maltese honey was tested against *Escherichia coli* ATCC® 25922, *Staphylococcus aureus* ATCC® 259213 and *Pseudomonas aeruginosa* ATCC® 27853. The medium of choice for broth dilution testing was cation-adjusted MHB (CAMHB). The activity was compared against artificial honey so as to show whether the activity is merely the result of the high osmotic potential of honey. This was prepared by dissolving 81 g D-fructose, 67 g D-glucose, 15 g maltose and 3 g sucrose in 34 ml filter sterilised distilled water [15]. A broth macro-dilution assay was used to analyse the honey and control samples [16]. 1 ml of each honey sample and artificial honey (0.0625–1.0 g/ml) and 1 ml of inoculum suspension (5×10^5 CFU/ml) were incubated for 20 hours at 35°C. The minimum inhibitory concentration (in g/ml) was then obtained.

2.15. Statistical analysis

Statistical significance was set at $p < 0.05$. One-way ANOVA with Bonferroni post-hoc test was conducted on all the parameters studied to compare between the seasons. These were performed with GraphPad Prism ver.5.0 for Windows (San Diego, CA, USA). The parameters were then analysed with multivariate analysis of all the honey samples. The correlation matrix was calculated, giving the correlation coefficients between each pair of variables present. To identify variability and to reduce the dimensions of the dataset, principal component analysis (PCA) was performed, using the XLSTAT Version 2011.5.01 software (Addinsoft, New York, NY, USA).

3. Results and discussion

3.1. Honey sampling

According to the population density map (**Figure 2**), the Western and Northern Districts are the least populated on the island of Malta. This reflects the highest vegetation density in these two districts when compared to the others. The Northern district shows a high sample percentage during the summer season (37%) as compared to the autumn and spring seasons (17 and 21%, respectively).

3.2. Physical appearance: colour and consistency

The results obtained for the colour and consistency of honey are illustrated in **Table 2**, and **Figure 3** and **Figure 4**, respectively. The darkest colour was observed for the autumn honey.

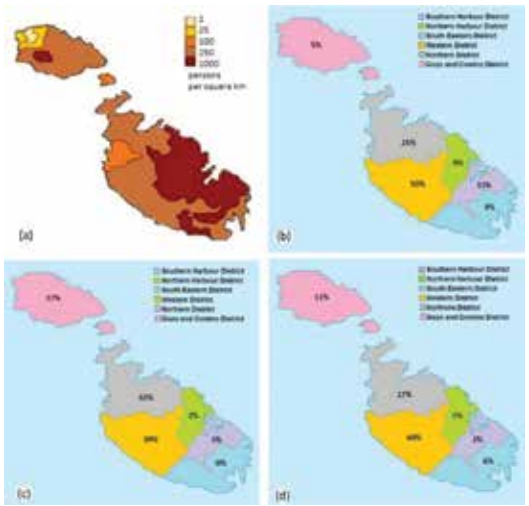

Figure 2. Spatial maps for (a) population density (adopted with permission from [17]) (b) spring, (c) summer and (d) autumn honey sample collection.

	Summer	Autumn	Spring
Colour (560 nm)	1.844 ± 0.242	3.909 ± 0.207***	2.143 ± 0.299
Consistency	2.563 ± 0.190	2.227 ± 0.254	1.750 ± 0.083**

**p < 0.01.
***p < 0.001.

Table 2. Mean absorbance values at 560 nm and mean consistency values for honey samples from the three seasons.

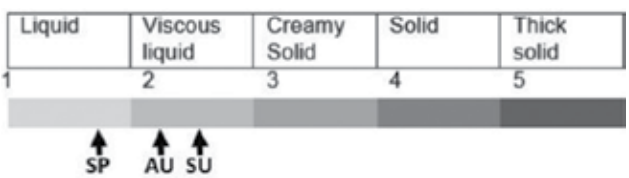

Figure 3. The colour scale for honey samples from the three honey seasons. SU = summer, SP = spring, AU = autumn.

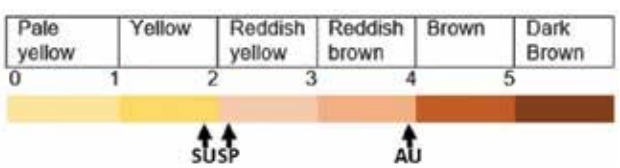

Figure 4. The consistency scale for honey samples from the three honey seasons. SU = summer, SP = spring, AU = autumn.

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The autumn honey is characterised by carob and eucalyptus sources. This honey is so distinctive, compared to other honey types ($p < 0.001$), that it is sometimes confused with carob syrup. Spring honey has a more liquid consistency than honey from the other two seasons ($p < 0.01$). As the nectar type determines seasonality, this has no direct impact on the consistency of honey and therefore some other factor might influence this parameter. This can only be determined through the investigation of other physicochemical characteristics.

3.3. Determination of brix and moisture content in honey

The brix and moisture contents of honey are illustrated in **Table 3**. Although in general there are minimal differences between the brix values for each particular season with year, seasonal statistical analysis reveals a significantly lower brix values for the autumn honey samples as compared to the other two seasons, that is, less than 79.90% (autumn) as opposed to more than 80.33% (spring and summer). The moisture content is practically opposite to the brix value, in which case autumn honey moisture content is significantly higher ($p < 0.001$) than that of the other two seasons. That is, more than 18.52% (autumn) and opposed to less than 18.46% (spring and summer). The main reason for this difference may be due to the abundance of water during the beginning of autumn, which is considered as the rainy season. Although during winter it is likely that 'winter honey' is produced, this is removed before the spring season starts, as this honey is mainly made from syrup. This is mandatory as syrup honey is considered as adulterated honey. The use of syrup during the winter months is only allowed so as to maintain the bee colony alive and healthy, considering that during winter very few plant species flower.

	2011		2012		2013		2014	
	Brix (%)	Moisture (%)	Brix (%)	Moisture (%)	Brix (%)	Moisture (%)	Brix (%)	Moisture (%)
Spring	80.88 ± 0.33	17.68 ± 0.33	81.02 ± 0.15	17.29 ± 0.16	80.33 ± 0.37	17.91 ± 0.43	80.40 ± 0.23	17.81 ± 0.22
Summer	80.26 ± 0.20	18.46 ± 0.16	80.63 ± 0.22	17.72 ± 0.24	81.20 ± 0.38	16.88 ± 0.33	80.76 ± 0.16	17.49 ± 0.16
Autumn***	79.20 ± 0.27	19.20 ± 0.24	79.90 ± 0.36	18.52 ± 0.42	76.69 ± 0.67	20.69 ± 0.88	79.15 ± 0.68	19.23 ± 0.71

***p > 0.001.

Table 3. Mean percentage brix and moisture values for honey samples from the three seasons between 2011 and 2014.

3.4. Determination of pH and free acidity of honey

The acidic nature of honey is important for several reasons. The most important reason is that the low pH inhibits the presence and growth of microorganisms. Other aspects of food technology, permit the honey to be blended with other food products, due to its low pH. The acidic nature also contributed to the flavour of honey particularly in monofloral honeys [18]. **Table 4** shows the mean pH and mean acidity values for honey samples from the three seasons. It was observed that there is slight yearly variation between mean pH values for the separate seasons. However, seasonal statistics reveal significant differences between the three season, the pH being the highest for the autumn honey (pH > 3.95), followed by summer honey (pH < 3.95) and finally spring (pH < 3.84). On the other hand, the total acidity was not statistically different for the three seasons, meaning that the organic

	2011		2012		2013		2014	
	pH	Acidity	pH	Acidity	pH	Acidity	pH	Acidity
Spring**	3.77 ± 0.03	32.55 ± 1.39	3.75 ± 0.02	34.12 ± 1.38	3.73 ± 0.08	41.13 ± 5.39	3.84 ± 0.03	33.29 ± 1.16
Summer	3.84 ± 0.03	34.74 ± 1.16	3.87 ± 0.06	45.52 ± 2.82	3.73 ± 0.11	40.04 ± 4.12	3.95 ± 0.02	29.71 ± 1.53
Autumn***	4.01 ± 0.04	29.48 ± 2.77	4.04 ± 0.07	43.53 ± 4.73	3.95 ± 0.03	40.85 ± 2.76	3.98 ± 0.09	30.68 ± 3.02

**$p < 0.01$.
***$p > 0.001$ for pH values.

Table 4. Mean pH and mean acidity (mM/kg) values for honey samples from the three seasons.

acid content did not seem to differ of these three seasons. pH mirrors the moisture content of the seasonal honeys. This may reflect the mobility of more free protons (H^+) with a higher moisture content.

3.5. Determination of electrical conductivity

The electrical conductivity of honey is measured at 20°C using a 20% solution of honey on dry weight basis. Conductivity is measured in mS/cm or μS/cm, reflecting the presence of ionizable substances, such as minerals [19], typically not exceeding 800 μS/cm. **Table 5** shows the mean conductivity values for honey samples from the three seasons throughout the project period. It was observed that there is slight yearly variation between mean pH values for the separate seasons. However, seasonal variations were significant. Autumn honey has the highest and a significantly different conductivity of all three seasons (EC_{autumn}>963.6 μS/cm compared to the other two seasons (<752.5 and <767.1 μS/cm for summer and spring, respectively). The high salt content for autumn honeys may occur due to the arid summer conditions that result in the salting out of minerals during this period (summer). When precipitation commences in autumn, the high salt content is dissolved leading to a higher uptake in plants, and the accumulation of salt in the nectar. The salt accumulation on autumn plants following a dry period was observed in other studies under local conditions [20].

3.6. The determination of HMF after White

5-Hydroxymethylfurfural (5-HMF) is an aldehyde, which can be used as an indicator of honey quality deterioration. 5-HMF forms through the Maillard reaction, a complex series of reactions between amino acids and reducing sugars (hexoses). The International Honey Commission [7] recommends three methods for the determination of HMF. The method described by White [8] involves the measurement of UV absorbance of clarified aqueous honey solutions with and without bisulphite. An HPLC method is also described in the IHC harmonized methods [7].

The Codex Alimentarius [21] established that processed or blended honey should not contain HMF levels higher than 80 mg/kg. The European Union [6] adopted the same upper limit for honey coming from Countries or Regions with tropical temperatures. In most cases, an upper limit of 40 mg/kg is applicable in EU member states.

Table 5 shows the mean HMF values for honey samples from the three seasons throughout the project period. HMF was exceptionally higher in autumn samples as opposed to summer and

	2011	2012	2013	2014
	Mean conductivity (μS/cm) values			
Spring	669.00 ± 54.11	569.30 ± 40.19	590.80 ± 85.45	767.10 ± 24.27
Summer	739.40 ± 73.97	685.50 ± 68.13	708.00 ± 108.60	752.50 ± 26.35
Autumn***	1895.00 ± 143.60	1296.00 ± 146.70	963.60 ± 87.18	1028.00 ± 190.10
	Mean HMF (mg/kg) values			
Spring	8.57 ± 2.02	27.60 ± 6.02	12.56 ± 2.74	10.85 ± 2.40
Summer	16.91 ± 5.80	36.15 ± 6.13	18.60 ± 2.94	25.11 ± 1.76
Autumn*	27.50 ± 7.71	59.50 ± 17.79	15.80 ± 3.01	16.83 ± 1.14
	Mean diastase (Schade units) values			
Spring	4.84 ± 0.66	9.10 ± 0.79	11.35 ± 0.49	11.55 ± 1.00
Summer	5.77 ± 0.41	10.89 ± 0.94	2.98 ± 1.06	8.62 ± 0.65
Autumn**	5.27 ± 0.62	7.87 ± 1.78	6.54 ± 0.51	8.70 ± 0.33
	Mean proline (g/kg) values			
Spring	0.28 ± 0.06	0.66 ± 0.13	0.67 ± 0.02	0.57 ± 0.06
Summer	0.24 ± 0.02	0.71 ± 0.05	0.16 ± 0.04	0.57 ± 0.05
Autumn	0.32 ± 0.03	0.78 ± 0.08	0.25 ± 0.02	0.69 ± 0.09

*$p < 0.05$;
**$p < 0.01$.
***$p < 0.001$.

Table 5. The mean conductivity (μS/cm), mean HMF (mg/kg), mean diastase (Schade units) and mean proline (g/kg) values for honey samples from the three seasons.

spring honeys ($p< 0.05$). It was observed that 2012 honeys from all three seasons exhibited higher HMF content with respect to other years. It was expected that summer honey may contain more HMF. However, with a higher brix level and lower water content, the HMF production is favoured. Honey samples turn darker (browner) in colour due to the accumulation of HMF.

3.7. The determination of diastase activity

Diastase, also referred to as any α-, β- or γ-amylase, can break down carbohydrates. Hence, diastase is the enzyme that converts the long chain starch to dextrins and sugars. This enzyme is produced by the bees and introduced into honey by the bees themselves. Diastase is used an indication of adulteration as honey that is harvested from hives which are feed sucrose to produce high volumes will have a diastase content which is low.

The α-amylase (alternative names: 1,4-α-D-glucan glucanohydrolase; glycogenase) is a calcium metalloenzyme, completely unable to function in the absence of calcium. As opposed to HMF, diastase activity decreases with time. However, this is another quality parameter where the degradation of honey enzymes indicates a decline in the functionality of the honey as a food supplement and also as a medicine.

Table 5 shows the mean diastase values for honey samples from the three seasons throughout the project period. Diastase was exceptionally lower in autumn samples (<8.70 Schade units) as opposed to spring honeys (>9.10 Schade units, $p < 0.01$). However, for 2011, the spring diastase level was low compared to the other years. The summer samples showed a varied diastase level, with the lowest values obtained during 2013 (2.98 Schade units) and highest values obtained during 2012 (10.89 Schade units).

Possible heating of honey to skim waxes should be avoided. Unfortunately this is a common local practice amongst beekeepers as the Maltese consumer prefers liquefied honey. It seems that enzymatic activity is more sensitive to heat than HMF and perhaps diastase activity may be considered as a more significant indicator of quality than HMF. However, diastase degradation seems to have less implications on human health than HMF accumulation.

3.8. The determination of proline

Honey is very low in protein. As a matter of fact it contains less than 1%. The protein portion is mainly made up of several amino acids. A chemical marker that represents proteins, i.e. amino acids, is proline. Proline is not an amino acid as there is no free amine group in its structure. Although proline content is not considered as one of the main indicators of honey quality, legal issues can be resolved by taking into consideration this parameter. A honey that contains less than 180 mg of proline per kilo of honey is an altered honey [7].

Table 5 shows that all mean proline values for honey from the three seasons (>240 mg/kg) were well above the 180 mg of proline per kilo of honey standard, except for the summer 2013 honeys (160 mg/kg). The years 2012 and 2014, showed a very high content of proline throughout the three seasons. Compared to the other two seasons, autumn 2012 and 2014 showed exceptionally high proline content. Combining all the 4 years for the three seasons, the highest proline content was observed for spring. However, the difference in proline contents was not significant with the other two seasons.

3.9. Determination of sugar content

Honey is made up of a matrix of sugars. Although the brix content provides a good indication of the content of sugars, the individual sugars are not identified by this method. Honey sugars are formed by the action of several honey bee enzymes on the floral nectar. The result is a complex mixture composed of 70% of monosaccharides and 10–15% disaccharides. Honey is also used as a sweetener in hundreds of products manufactured [22]. The oligosaccharide content of honey contributes to its prebiotic properties, promoting the growth of Bifidobacteria and Lactobacilli [23]. Many scientists attempted to characterize the sugars in many honey types [24–26]. Different techniques such as HPLC [27, 28] or GC-MS [29] were used. These methods have been standardized by the "International Honey Commission" [7]. The HPAEC-PAD (high-performance anion-exchange chromatography with pulsed amperometric detection) is one of the most used techniques for the analysis oligosaccharides [30].

In some studies [31, 32], the disaccharide content was used to characterize the type and origin of the honey. Moreover, maltose, turanose and nigerose were useful for differentiating Brazilian honey in several other geographic areas [33]. The sugar profiles are also used to

	Fru	Glu	Fru+Glu	Suc	Mal	Mel
Spring	44.07 ± 0.27	40.56 ± 0.29	84.62***	11.69 ± 0.50	3.58 ± 0.19	0.00 ± 0.00
Summer	47.19 ± 1.06	39.89 ± 0.66	87.08**	8.45 ± 0.73	4.13 ± 0.11	0.00 ± 0.00
Autumn	48.64 ± 0.82	41.90 ± 0.71	90.54	5.41 ± 0.51***	3.66 ± 0.27	0.38 ± 0.38

Fru, fructose; Glu, glucose; Suc, sucrose; Mal, maltose; Mel, melezitose
**$p < 0.01$.
***$p < 0.001$.

Table 6. Mean percentage sugar values for honey samples from the three seasons throughout the project period.

differentiate honeydew honey from flowers. Indeed, the honeydew presents lower concentrations of glucose and fructose and higher oligosaccharides including melezitose or erlose [34]. It is worth noting that the concentrations of fructose and glucose are used to classify floral honey [7]. Two disaccharides of importance are sucrose and maltose. According to Council Directive [6], the content should not exceed 5% of the total sugars.

Table 6 provides the mean percentage sugar values for honey samples from the three seasons throughout the 4-year period. **Figure 5** shows a typical chromatogram for the Maltese honey samples. The most abundant sugar is fructose followed by glucose. The disaccharides, sucrose and maltose, are found in lower concentrations. Maltose is more consistent than sucrose. Melezitose is only present in autumn honey. The content of glucose and fructose for the three seasons, as shown in **Table 6**, is well above the content stated in Ref. [5], which is a minimum of 60 g/100 g. For the three honey types the amount exceeded 84 g/100 g.

Fructose is significantly high in autumn honey (48.64%) and least in spring honey (44.07%). Glucose is relatively similar in all seasonal honey types ranging from 39.89% for summer honey to 41.90% for autumn honey. Sucrose varies significantly between the three seasons; in spring honey being the highest (11.69%) and in autumn honey being the lowest (5.41%). This goes in accordance with the brix results, which partially concluded the possible feeding of a sucrose syrup to bees during winter, when flora is scarce, and the possible incorporation of this 'syrup honey' within the spring honey. Therefore, beekeepers should be advised that the honey produced from the artificial syrup should be discarded prior to the commencement of honey production in spring. Melezitose (**Table 6**) was only present in autumn honey. However, not all autumn honey samples contained this saccharide. This sugar is typical of honeydew honey. Honeydew is a sugar-rich sticky liquid,

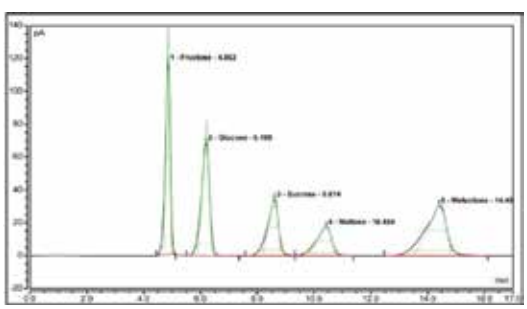

Figure 5. A typical chromatogram for honey samples.

produced mainly by aphids and some scale insects as they feed on plant sap. According to Mifsud et al. [35], aphids are present on carob and citrus during autumn, particularly *Aphis* (Aphis) *gossypii* on carob, *Aphis* (Aphis) *craccivora* and *Toxopteraaurantii* on citrus. This may explain the presence of honeydew honey within the floral honey during autumn. Honeydew honey may be produced during summer and autumn [36]. However, due to limitations in tree numbers, this cannot be produced on a large scale in Malta. Therefore, a possible indicator of pure autumn honey may be melezitose, although this needs to be further investigated.

3.10. Polyphenolic content

The mean total polyphenolic content in mg TAE/100 g honey for spring (56.943 ± 7.027) was significantly lower ($p < 0.05$) than for the other two seasons (AU: 79.692 ± 8.000 and SU: 69.598 ± 3.208 mg TAE/100 g honey) (**Table 7**). It was observed that the darker the honey colour, the higher was the total polyphenolic content. This was the case with autumn honey samples [12].

3.11. Total flavonoid content

In spite of the higher flavonoid content for some autumn honey samples (212.86 and 197.57 mg RE/100 g), there was no statistical difference in content for honey samples from the three seasons (**Table 7**). The mean flavonoid content for the three seasons was 31.154 ± 17.729, 37.651 ± 8.460, and 31.420 ± 11.373 mg RE/100 g honey, respectively. As compared to similar studies, the Maltese honey samples contained superior flavonoid content to that observed in other similar studies with quantities ranging between 1.35 and 9.78 mg RE/100 g honey [37, 38].

3.12. Radical scavenging activity: the DPPH assay

The mean DPPH inhibition in mg AEAC/100 g honey for autumn, spring and summer was 9.300 ± 1.292, 5.805 ± 0.610 and 5.238 ± 0.657, respectively (**Table 7**). The autumn honey samples had a superior DPPH inhibitory activity with respect to the other two seasons ($p < 0.01$). This may be due to the presence of carob nectar in autumn honey which contains high amounts of polyphenols and tannins, as noted in Ref. [39].

	TPC	TF	DPPH	Red Pow
Spring	56.943 ± 7.027*	37.651 ± 8.460	5.805 ± 0.610	16.600 ± 1.979
Summer	69.598 ± 3.208	31.420 ± 11.373	5.238 ± 0.657	14.250 ± 0.035
Autumn	79.692 ± 8.000	31.154 ± 17.729	9.300 ± 1.292**	12.67 ± 1.093

*$p < 0.05$.
**$p < 0.01$.

Table 7. Total polyphenolic (mg TAE/100 g honey), total flavonoid (mg RE/100 g honey), DPPH (AAE-DPPH mg AEAC/100 g honey) and reducing power (mg AEAC/100 g honey) values for honey samples from the three seasons.

3.13. Reducing power

The Maltese honey samples had antiradical activity values between 3.33 and 15.62 mg AEAC/100 g honey (**Table 7**). The mean reducing power in mg AEAC/100 g honey for the

autumn, spring and summer seasons were 12.67 ± 1.093, 16.600 ± 1.979 and 14.250 ± 0.035 mg AEAC/100 g, respectively. The reducing power values were similar to those obtained by Savatović et al. [38], i.e. 1.43–7.82 mg AEAC/100 g honey but lower than those obtained by Meda et al. [40], i.e. 10.20–37.87 mg AEAC/100 g honey. In the study by Savatović et al. [38], it was pointed out that monofloral honeys provide a higher reducing power than multifloral honeys. This was also observed for the Maltese honey with the monofloral autumn and summer samples showing higher activity (7.54 and 6.96 mg AEAC/100 g honey) than the multifloral spring samples (5.98 mg AEAC/100 g honey).

3.14. Antimicrobial activity

Maltese honey exhibited MIC values ranging between 0.067 and 0.205 g/ml (**Table 8**). In spite of the statistical insignificance, the spring honey samples showed the best MIC values compared to the other two seasons. The honey samples were compared against artificial honey as highlighted earlier. Only spring samples against *S. aureus* and *P. aeruginosa* showed a significantly lower MIC than the artificial honey ($p< 0.001$ and $p< 0.05$, respectively) [41]. *S. aureus* strains are known to be involved in acquired and nosocomial infections, while *P. aeruginosa* may cause diabetic ulcers, wound infections and urinary tract infections [42]. Therefore, Maltese honey may be potentially useful for the topical treatment of microbial infections particularly associated with wounds and ulcers.

	Spring	Summer	Autumn	Artificial honey
E. coli	0.165 ± 0.0255	0.172 ± 0.0392	0.203 ± 0.0468	0.250 ± 0.0000
S. aureus	0.067 ± 0.0162***	0.125 ± 0.0255	0.157 ± 0.0182	0.250 ± 0.0000
P. aeruginosa	0.110 ± 0.0155*	0.157 ± 0.0403	0.172 ± 0.0202	0.250 ± 0.0000

*p < 0.05.
***p < 0.001.

Table 8. Total minimum inhibitory concentrations (g/ml) for honey samples from the three seasons.

3.15. PCA analysis of physicochemical parameters and sugar content

It was observed from the scree plot that the first three components accounted for 50.31% of the total variance. However, the parameters studied fall within different components. The scores plot (**Figure 6**) shows the physicochemical parameters of honey samples in the space of the two new variables, F1 and F2. The parameters plot shows that brix and moisture are inversely related, while acidity, diastase and proline are particularly inversely related to HMF and pH.

Moving along F1, it was observed that the honey samples were distributed by those with low moisture and brix contents on the left and those with the highest moisture and brix contents on the right. From left to right, the samples moved from summer to spring to autumn.

To determine any possible clustering for the seasonal honey, the sugar content was used subjected to principal component analysis. It was observed from the scree plot that the first two components accounted for 71.843% of the total variance. The parameters studied fell within the first two components. The scores plot (**Figure 7**) shows the sugar content of honey samples in the space of the two new variables, F1 and F2. The parameters were grouped as factor 1 for the most common sugars in honey (fructose, glucose and sucrose). This analysis shows that fructose and glucose are inversely related to sucrose.

Moving along F1, it was observed that the honey samples were distributed by those with a high fructose and glucose and low sucrose on the left and those with the lower fructose and

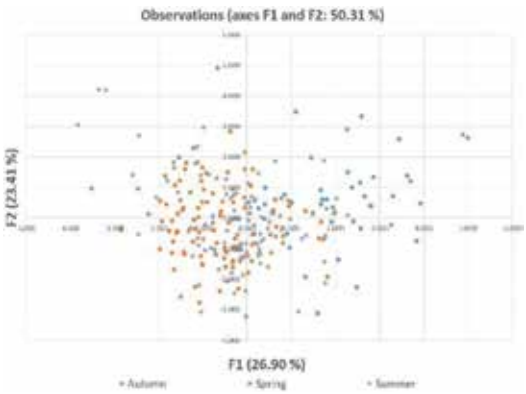

Figure 6. Score plot of seasonal honey analysed by PCA (physicochemical).

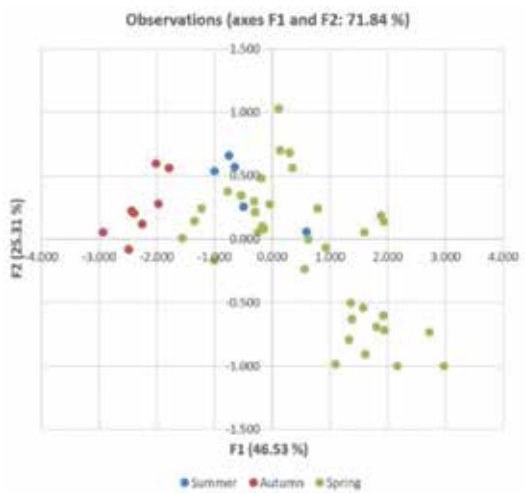

Figure 7. Score plot of seasonal honey analysed by PCA (sugars).

glucose and higher sucrose on the right. From left to right, the samples moved from autumn to summer to spring. Autumn honey samples were particularly distinctive from the other two seasonal honey types. This may be due to the fact that autumn honey samples contained melezitose as opposed to the other two types.

3.16. Concluding remarks on Maltese honey

The physicochemical characterisation of Maltese honey was conducted over a period of 4 years, in order to determine any typical similarities and differences that may be attributed to the different seasonal characteristics. The main characteristics are meteorological conditions that typify the season and the seasonal floral diversity. Data is not shown for the latter parameter, as the floral distribution is beyond the scope of this present study. The typical characteristics of seasonal Maltese honey are as follows.

Spring honey is typically reddish yellow in colour with a liquid consistency. It may be classified as a multifloral honey, featuring nectar and pollens from a vast number of plant that flower during spring. Summer honey is usually yellowish coloured with a viscous consistency. This typically features thyme due to the translocation of hives to areas (North of Malta) rich in thyme during early summer. Autumn honey is dark (reddish-brown) in colour and may contain honeydew due to the tree-related nectars. Therefore, it may contain Melezitose as a minor sugar. It usually has a higher conductivity in relation to the other seasonal honeys, but HMF tends to be high too. This typically contains carob and eucalyptus nectar and pollens. **Table 9** illustrates the typical physicochemical parameters for the three seasonal honey types. In conclusion, **Figure 8** shows a radial plot for the three seasonal honey-types.

Test	Range*	Spring honey	Summer honey	Autumn honey
Moisture (%)	<20	17.29-17.91	16.88-18.46	18.52-20.69
Brix (%)	≈80	80.33-81.02	80.26-81.20	76.69-79.90
Conductivity (μS/cm)	<800	569-767	685-752	963-1895
pH	3-5.5	3.7-3.8	3.7-3.9	3.9-4.0
Acidity (mM/kg)	8.7-46.8	32.5-41.1	29.7-45.5	29.5-43.5
HMF (mg/kg)	<40	9-28	17-36	16-60
Proline (mg/kg)	>180	280-670	160-710	250-780
Diastase activity (Schade units)	>8	5-12	3-11	7-9
Fructose and glucose (%)	>60	≈85	≈87	≈91
Sucrose (%)	<5	≈12	≈8	≈5

*According to Ref. [6]

Table 9. Typical ranges for physicochemical parameter for spring honey.

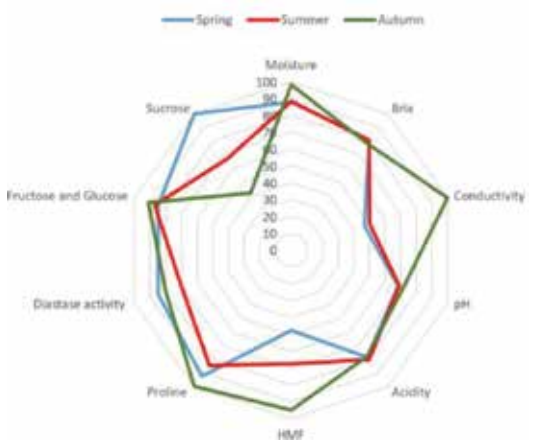

Figure 8. A radial plot for seasonal honey.

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Chapter 9

Techniques for the Evaluation of Physicochemical Quality and Bioactive Compounds in Honey

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Additional information is available at the end of the chapter

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Abstract

Honey is a concentrated aqueous solution of sugar, especially glucose and fructose, and minor amounts of dextrin, enzymes, waxes, volatile oils, organic acids, ethers, albuminoidal gum substances and minerals. Commercially available honey samples vary in quality according to various factors such as climate diversity, type of flora of the surrounding region, geographical characteristics, processing, floral supply period, and packaging and storage conditions, which can compromise the standardization and quality of the final product. The different techniques that will be presented in this chapter to assess the quality of honey are tests required by identification standards and national and international quality control or are important quality tools that can be used in the evaluation of the conditions for obtaining and processing of the honey, fraud identification and changes to and/or adulteration of the honey, ensuring the physical and chemical composition of the project and guaranteeing quality standards, directly impacting the shelf life and use and presentation of the product.

Keywords: physicochemical characterization, honey quality standards, antioxidant activity, total polyphenols, flavonoids

1. Introduction

Honey is produced by honeybees from the nectar of flowers or from secretions from the living parts of plants or from vegetable sap by sucking insects that remain on the living parts of the plant. Bees collect and transform this material with their own specific substances before

storing it and leaving it to mature in separate honeycombs [1–3]. Honey is characterized as a semi-liquid product, comprising a complex mixture of carbohydrates, especially the monosaccharides glucose and fructose; and other sugars, enzymes, lactones, wax, pigments, vitamins, amino acids, minerals, organic acids and pollen [4]. Its chemical composition varies according to the bee species, weather conditions, type of soil, physiological state of the colony, nectar source and honey maturity [5]. Its nutritional quality, which occurs due to the presence of minerals and vitamins, sensory properties, medicinal properties such as antioxidant and antiseptic activity, specific therapeutic properties, such as for the treatment of inflammatory and infectious processes, and high energy content attract many consumers [6, 7].

In Brazil, Normative Ruling No. 11 of October 20, 2000, which regulates the standardization of honey for marketing purposes, is based on European laws and approves only honey produced by bees of the Apis genus [3]. The physicochemical analyzes indicated by Brazilian legislation for the identity and quality of honey produced by bees from the Apis genus are moisture, sucrose, reducing sugars, ash, minerals, acidity, diastase activity, color and hydroxymethylfurfural (HMF) content [3]. These analyses contribute to the supervision and control of the quality of honey produced in Brazil and intended for export, and the results are compared with both Brazilian standards and those of international organizations [5, 8], see **Table 1**. Some concerns exist regarding the quality of domestically produced honey, and such tests allow the quality of imported honey to be inspected [5].

The aim of this chapter is to significantly contribute to the improvement of techniques that evaluate the quality of honey, and to propose an adjustment to the physicochemical parameters established by Brazilian law [3], adding additional analysis such as pH, formaldehyde index (mL kg^{-1}), electric conductivity (mS cm^{-1}), protein (%), total reducing sugars (%), viscosity (mPa s) and water activity. These analyzes can contribute effectively to control the quality of commercially available honey. The analysis required by existing legislation combined with

Parameters	Brazil (2000)	Mercosur (1999)	European Union (2001)
Moisture (%)	Maximum 20.0	Maximum 20.0	Maximum 20.0
Acidity (meq kg^{-1})	Maximum 50.0	Maximum 50.0	Maximum 50.0
Ash (%)	Maximum 0.6	Maximum 0.6	–
Color	Nearly colorless to dark brown	Nearly colorless to dark brown	–
HMF (mg kg^{-1})	Maximum 60.0	Maximum 60.0	Maximum 60.0
Electric conductivity (μS cm^{-1})	–	–	Maximum 0.8
Reducing sugars (%)	Minimum 65.0	Minimum 65.0	Minimum 60.0
Saccharose (%)	Maximum 6.0	Maximum 6.0	Maximum 5.0
Diastase activity (Goethe)	Minimum 8.0	Minimum 8.0	Minimum 8.0

Source: Brazil [3]; Mercosur [9] and European Union [10].

Table 1. National and international standards for honey from *Apis mellifera* L.

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the further analysis proposed by this chapter will allow important parameters for the quality of honey to be determined, such as maturity, purity, deterioration and adulteration.

2. Methods

All analyses were performed in triplicate to provide greater reliability for the results, following the methods described in the below sections.

2.1. Moisture

Water content is one of honey's most important characteristics as it influences its viscosity, specific gravity, maturity, crystallization, flavor, preservation, shelf life and palatability [11–13]. It depends on several factors such as bee species, floral source, honey harvesting time, the degree of maturity achieved in the hive (complete dehydration) and climatic factors [14].

Moisture is analyzed to determine the safety of the product, giving a quality criterion that determines the ability of the honey to remain stable and free of fermentation. A high moisture content can lead to crystallization of the product and promote the development of osmophilic microorganisms responsible for fermentation, negatively affecting its sensory characteristics and nutritional properties and reducing the shelf life of the product [15].

Method: humidity is determined according to the method described by the Atago Co [16], using the refractometric method. The method is based on the relationship between the speed of light in a vacuum and a substance through which an incidental beam of light is passed, through a honey solution [8, 16]. This device is adapted from the Abbe refractometer and features a scale, which expresses the value in brix, from which the humidity value is calculated. Three drops of honey are placed in the refractor device and, after adjusting the angle limit, the reading of the refractive index is taken directly from the scale [8]. The refractive measurement provides the dry matter content in all cases where there are pure sugar solutions. When the sugar solution is mixed with other substances, such as honey, the value found is usually very close to the total for the dry matter [17]. Therefore, to obtain the moisture from the honey, the value of the refractive index is checked with a correlation table showing the relationship between the refractive index and the moisture of the honey (**Table 1**). **Table 2** presents the result of an equation developed by Wedmore from the data of Chataway [15].

The refractive index of liquids is also temperature dependent. Generally, refractometers are regulated at 20°C [17]. If the temperature of honey is exactly 20°C, the refractive index obtained directly from **Table 1** can be applied. However, for measurement at different temperatures, the refractive index should be increased or decreased by a value of 0.00023 for each degree Celsius above or below 20°C, depending on the sample temperature. In the case of refractive index values not included in **Table 2**, the desired value can be calculated using Eq. (1).

$$y = 614 \times 60 - 400 \times x, \quad (1)$$

where y = moisture, x = refractive index.

Refractive index (20°C)	Moisture (%)	Refractive index (20°C)	Moisture (%)
1.4740	25.0	1.4865	20.0
1.4745	24.8	1.4870	19.8
1.4750	24.6	1.4875	19.6
1.4755	24.4	1.4880	19.4
1.4760	24.2	1.4885	19.2
1.4765	24.0	1.4890	19.0
1.4770	23.8	1.4895	18.8
1.4775	23.6	1.4900	18.6
1.4780	23.4	1.4905	18.4
1.4785	23.2	1.4910	18.2
1.4790	23.0	1.4915	18.0
1.4795	22.8	1.4920	17.8
1.4800	22.6	1.4925	17.6
1.4805	22.4	1.4930	17.4
1.4810	22.2	1.4935	17.2
1.4815	22.0	1.4940	17.0
1.4820	21.8	1.4946	16.8
1.4825	21.6	1.4951	16.6
1.4830	21.4	1.4956	16.4
1.4835	21.2	1.4961	16.2
1.4840	21.0	1.4966	16.0
1.4845	20.8	1.4971	15.8
1.4850	20.6	1.4976	15.6
1.4855	20.4	1.4982	15.4
1.4860	20.2	1.4987	15.2

Source: Bogdanov et al. [15] and AOAC [18].

Table 2. Determination of honey moisture from the refractive index.

2.2. pH

The pH determined refers to the hydrogen ions present in a solution of honey and can influence the formation of other components such as the production of hydroxymethylfurfural—HMF [19].While pH analysis is useful as an auxiliary variable to estimate the quality of the product and as a parameter for evaluating total acidity, it is not directly related to free acidity due to the actions of the buffer acids and minerals present in honey [20].

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The pH of honey ranges between 3.5 and 5.5 depending on its botanical source, the pH of nectar, soil or plant association, and the concentration of different acids and minerals such as calcium, sodium, potassium and other ash constituents [2, 15]. Altered values may indicate fermentation or adulteration [12, 21]. Mandibular substances added to the nectar may also change the pH of honey, a process that begins with the transport of nectar to the hive in the honey vesicle [22].

Method: pH is determined according to the method described by De Moraes and Teixeira [23]. Weigh 10 g of honey in a 100 mL beaker using an analytical balance and homogenize the sample in 75 mL of distilled water. Thereafter, using a pH meter calibrated with appropriate buffers (pH solution of 7.0 and 4.0 pH solution) for each honey sample, a direct reading is taken from the device.

2.3. Acidity

Due to the variations of some organic acids and inorganic ions such as phosphate and based on different sources of nectar, honey acidity can result from the action of the enzyme glucose oxidase produced in the hypopharyngeal glands of bees, producing gluconic acid. This enzyme remains active even during storage affecting the honey after processing due to the quantity of minerals present, and by bacteria during maturation [15, 24, 25]. Organic acids from honey represent less than 0.5% of solids, but have a considerable effect on taste [26].

Method: acidity is determined in accordance with the method described by De Moraes and Teixeira [23]. Weigh 10 g of honey in a 100 mL beaker with an analytical balance; homogenize the sample in 75 mL of distilled water; add five drops of alcoholic solution of phenolphthalein. With the aid of a pH meter and a magnetic stirrer, titration is slowly carried out with sodium hydroxide (NaOH) 0.1 N, until the solution reaches a pH of 8.5. Add 10 mL of sodium hydroxide (NaOH) 0.1 N to the sample to increase the pH to approximately 10. Titrate with hydrochloric acid (HCl) 0.1 N to slowly return the pH to 8.3. Note the volumes spent during each titration to calculate the total acidity of the sample. Acidity value is determined by Eqs. (2)–(4) and corrections of HCl and NaOH should be carried out in accordance with Eqs. (5) and (6).

$$\text{Free acidity : corrected volume of NaOH spent} \times 10, \tag{2}$$

$$\text{Lactonic acidity : (10 - corrected volume of HCl spent)} \times 10, \tag{3}$$

$$\text{Total acidity : free acidity + lactonic acidity,} \tag{4}$$

$$\text{HCl corrected} = \text{volume of HCl spent} \times \text{correction factor (fc),} \tag{5}$$

$$\text{NaOH corrected} = \text{volume of NaOH spent} \times \text{correction factor (fc).} \tag{6}$$

2.4. Formaldehyde content

The formaldehyde content in honey represents, predominantly, amino compounds, allowing the evaluation of peptide content, protein and amino acids [27]. This is an indicative of the presence of nitrogen in honey and is an important adulteration indicator. When low, it can suggest the presence of artificial products, while when excessively high it can show that the bees were fed hydrolyzed protein [28]. Thus, formaldehyde content can be used to prove the authenticity of honey [21].

Method: formaldehyde content is determined according to Moraes [29]. After performing the procedure for determining acidity when the pH of the sample reaches 8.3, the pH is reduced to 8.0 with two drops of 0.1 N acetic acid, and then 5 mL of 35% formalin is added to the sample. After one minute of agitation, the solution is titrated with sodium hydroxide (NaOH) 0.1 N, slowly returning the pH to 8.0. The volume of sodium hydroxide spent from the last titration is noted and the formaldehyde index is calculated in accordance with Eq. (7).

$$\text{Formaldehyde content} = \text{corrected volume of NaOH 0.1 N spent} \times 10 (\text{mL kg}^{-1}). \quad (7)$$

2.5. Ash

Ash content expresses the richness of honey in mineral content [30–32]. The minerals calcium (Ca), magnesium (Mg), iron (Fe), copper (Cu), cadmium (Cd) and zinc (Zn) in the form of sulfate (SO_4^{2-}) and chloride (Cl^-) [24] are found in small amounts. Minerals influence the color of honey and are present in higher concentrations in dark honey than light-colored honey [14]. They vary depending on the floral origin, region, bee species and type of manipulation [15].

Method: the method used is proposed by Marchini et al. [8] and C.A.C. [33] and is based on the weight loss that occurs when the product is incinerated to a maximum of 550°C, resulting in the destruction of the organic matter without changing the constituents of the mineral residue or causing loss by volatilization [8]. The crucibles are identified and heated in a furnace for approximately 25 min at 300°C. They are then transferred to the desiccator for 20 min to cool down. The crucibles are weighed separately with an analytical balance and the weights recorded. Approximately 10 g of sample is weighed, and the exact weight recorded. The samples are charred on an asbestos screen using a Bunsen burner until completely carbonized. They are then incinerated in an oven, raising the temperature gradually to 600°C. Wait for 5–7 hours until incineration is complete (white to light gray color). The still hot crucibles are removed from the oven and transferred to the desiccator. After 20 min the crucibles are weighed with an analytical balance and the weight recorded. The amount of ash is determined according to Eq. (8):

$$\text{Ash}(\%) = \left[\frac{m1 - m2}{m3}\right] \times 100, \quad (8)$$

where m1 = crucible weight with ashes, m2 = crucible weight, m3 = sample weight (mass of honey).

2.6. Electric conductivity

Electrical conductivity is determined by the ability of ions present in a solution to conduct electrons. It has been found to assist in the determination of the botanical origin of honey, as well as correlating with ash content, pH, acidity, minerals, proteins and other substances in honey [30, 34]. Honey conductivity is a great indicator of the adulteration of honey from its original form; whether formed from nectar (with some differentiation according to species) or honeydew [2].

Method: electrical conductivity is based on the fact that salt solutions conduct an electric current between two electrodes [35]. To measure this, a conductivity meter is used. After turning on the unit and waiting for it to stabilize; wash the ampoule of the equipment with distilled water and add a 1412 μS/cm buffer in order to calibrate the apparatus; then wait until the reading stabilizes.

Weigh 10 g of honey in a beaker on an analytical balance and transfer it to a 50 mL volumetric flask with distilled water. Take the reading as soon as the conductivity stabilizes. For each change of sample rinse the electrode with distilled water and dry it with absorbent paper.

2.7. Color

Color has a direct impact on the price of honey as it influences consumer preference and is of particular importance in the international market [8]. Variations in the color of honey are related to its floral origin, mineral content, storage and product processing, climatic factors during nectar flow and the temperature at which the honey matures in the hive [12], as well as factors such as the proportion of fructose and glucose present, nitrogen content and the instability of fructose in an acid solution [36].

Method: the evaluation of honey is based on the varying absorption of light of various wavelengths, depending upon the constituents present in the honey [19]. For the determination of color, a visible spectrophotometer is used. Select a wavelength of 560 nm; reset the tray of the machine using p.a. glycerin as a blank sample. Take the reading directly from the instrument display. Note the value and use the Pfund scale to determine the color according to range, in accordance with **Table 3**.

Color	Pfund scale (mm)*	Color range (inc)**
Water white	From 1 to 8	0.030 or less
Extra white	More than 8–17	More than 0.030–0.060
White	More than 17–34	More than 0.060–0.120
Extra light amber	More than 34–50	More than 0.120–0.188
Light amber	More than 50–85	More than 0.188–0.440
Amber	More than 85–114	More than 0.440–0.945
Dark amber	More than 114	More than 0.945

*Millimeter.
**Incidence—absorbance at 560 nm. Source: Marchini et al. [8].

Table 3. Pfund scale for determining color.

For this analysis, the honey must be liquid, without crystallization, as crystals tend to change the natural color of honey, making it lighter [8].

2.8. Hydroxymethylfurfural (HMF)

Hydroxymethylfurfural (HMF) is an intermediate product of the Maillard reaction, and is formed by the direct dehydration of sugars under acidic conditions, mainly by the decomposition of fructose during heat treatment applied to food [4, 30]. It can be a toxic compound when found in high amounts. In honey, HMF is an indicator of quality which assists in the identification of freshness when in low concentrations. Higher than permitted concentrations may mean that the product has undergone adulteration through the addition of inverted sugar (syrup), has been stored under inappropriate conditions, undergone prolonged storage, been heated, or affected by acidity, water or minerals [12, 36].

Method: the quantitative method proposed by Association of Official Analytical Chemists (AOAC) [18]. Prepare the following solutions:

Preparation of Carrez solution I: weigh 15 g of potassium ferrocyanide $K_4Fe(CN_6).3H_2O$ in an analytical balance; dissolve in distilled water and make up the solution in a 100 mL volumetric flask.

Preparation of Carrez solution II: weigh 30 g of zinc acetate $Zn(CH_3COO)_2.2H_2O$ in an analytical balance; dissolve in distilled water and make up the solution in a 100 mL volumetric flask.

Preparation of the sodium bisulfite solution $NaHSO_3$·0.2% (*m*/*v*): weigh 0.2 g of sodium bisulfite $NaHSO_3$ in an analytical balance; dissolve in distilled water and make up the solution in a 100 mL volumetric flask. Use this solution only on the day of preparation.

Weigh 5 g of honey in an analytical balance using a properly labeled 50 mL beaker, dissolve the sample by adding 25 mL of distilled water and then transfer it to a 50 mL volumetric flask. Add 0.5 mL of Carrez solution I and 0.5 mL of Carrez solution II and fill the volumetric flask to the meniscus with distilled water.

Add two drops of ethanol to prevent foaming. Mix the solution and filter using filter paper; discarding the first 10 mL filtered.

Label two test tubes and pipette 5 mL of the filtrate over 5 mL of distilled water in the first (sample) and 5 mL of the filtrate added to 5 mL of 0.2% sodium bisulfite solution in the second tube (blank). Shake the tubes using a vortex mixer. Measure the absorbance in a UV-vis spectrophotometer at wavelengths of 284 and 336 nm using quartz cuvettes.

Before the readings, calibrate the spectrophotometer with a blank reference for each sample evaluated. If absorbance at 284 nm exceeds 0.6 the sample is diluted with water and the blank reference with sodium bisulfite 0.2%, in the same proportions, and the reading is repeated. The HMF content in honey is calculated with Eq. (9) .

$$\text{HMF} = \frac{(\text{A284} - \text{A336}) \times 149.7 \times 5}{\text{Sample weight (g)}}, \tag{9}$$

where A284 = absorbance at 284 nm, A336 = absorbance at 336 nm, 149.7 = factor, 5 = theoretical value of sample weight.

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2.9. Protein

Despite little being known about the proteinaceous material present in honey, and its limited occurrence, such materials can be used to detect possible adulterations in commercial products, along with water content and concentration [37]. They are also used as identification parameters for the maturity of honey [38].

Honey protein can originate either from animals or plants. Animal protein comes from the bee itself, made up of secretions from the salivary glands, along with products collected during the collection of nectar or the maturation of the honey [11], while the plant origins are the nectar and pollen collected in the field [39].

Method: Determining the level of protein in honey is based on the modification of the nitrogen of the sample into ammonium sulfate through acid digestion, distillation and the subsequent release of ammonia, which is fixed in an acidic solution and titrated. Determining the nitrogen and the conversion factor provides the crude protein result, based on the Kjeldahl method and described by Silva and Queiroz [40].

Preparation of the catalytic mixture: weigh 10 g of sodium sulfate or anhydrous potassium and 1 g of copper sulfate pentahydrate. Grind in a mortar, mix thoroughly and store in a labeled flask.

Preparation of the sample: weigh 0.5 g of the sample on vellum. Then transfer the samples to the Kjeldahl tubes and add about 2.5 g of the catalyst mixture and 7 mL of p.a. sulfuric acid.

Digestion: place the labeled tubes in a block digester and gradually increase the temperature from 50 to 50°C to 400°C and maintain for 4–6 hours.

Distillation and neutralization: turn on the unit by checking the mains voltage and open the water tap to allow circulation in the condenser, observing the amount of water in the steam generation flask, which must be above the sensor. When necessary, complete using the water linking button. Turn the dial to 7/8 of the resistance to heat the steam generator and wait for the water to boil. Dissolve the sample in the digestion tube with 10 mL of distilled water; turn off the heat; take a 125 mL Erlenmeyer flask containing 15 mL of H_2BO_3 5% and add 5 drops of the mixed indicator—methyl red (0.1% in alcohol) and bromocresol green (0.1% in alcohol)—which is red for acidic and green for basic. Connect the digestion tube add approximately 20 mL of NaOH 50% to the hopper located above the equipment (the tap must be closed), and open the tap slowly until the sample is neutralized (becoming dark blue or dark brown). Around 15 mL was used; after neutralization is determined, close the soda tap funnel and turn on the heat button.

®*Titration*: prepare a burette with 50 mL of standard hydrochloric acid 0.01 M; titrate directly in the Erlenmeyer flask in which the distillate is placed. The end point of the titration is indicated by the solution changing color to pink. Perform the calculation according to Eq. (10).

$$\% \text{ Protein} = \frac{(V \times M \times fc \times 0.014 \times 100 \times 6.25)}{m}, \tag{10}$$

where V = volume of HCl spent in titration, M = molarity of hydrochloric acid, fc = correction factor of hydrochloric acid, 6.25 = correction factor for protein, m = sample weight.

2.10. Reducing sugars, total reducing sugars and sucrose

Sugars constitute 95% of the dry matter of honey [15], and together with water make up its main components. The monosaccharides glucose and fructose represent around 85% of the carbohydrates present in honey produced by the Apis genus, and are known as reducing sugars, which have the ability to reduce copper ions in an alkaline solution. Fructose has a high hygroscopicity and adds to the sweetness of honey, while glucose, due to its poor solubility, tends to influence crystallization [12]. Normally fructose is predominant as honey with high fructose rates can remain liquid for a long time, or never crystallize [2]. The disaccharides sucrose and maltose represent 10% of the sugars present in honey [41]. Sucrose represents on average 2–3% of the carbohydrates of honey from the Apis genus. When it exceeds this value, it indicates adulterated honey or early harvested honey, with humidity above 20% [19].

Method: This method is based on the ability of the reducing sugars glucose and fructose to reduce the copper present in a cupro-alkaline solution (Fehling's solution), characterized by the reduction of cupric ions to cuprous ions, and the oxidization of sugars into organic acids [8, 15].

Preparation of reagents: Fehling A: dissolve 34.65 g of p.a. copper sulfate pentahydrate ($CuSO_4.5H_2O$) in distilled water; transfer it to a 1000 mL volumetric flask and complete the volume. Fehling B: dissolve 125 g of p.a. sodium hydroxide (NaOH) in 300 mL of distilled water; in the same solution dissolve 173 g of p.a. tartrate of potassium and sodium ($C_4H_4KNaO_6.4H_2O$); complete the volume to 1000 mL and allow it to stand for 24 hours.

Standardization of Fehling's solution: weigh 0.5 g of p.a. glucose ($C_6H_{12}O_6$) pre-dried in an oven at about 70°C for 1 hour; transfer to a 100 mL volumetric flask using water. Dissolve well and adjust the volume. The standard glucose solution for the titration of the Fehling's solution should be prepared on the day of standardization. Place the standard glucose solution in the burette. Transfer 10 mL each of the Fehling Solutions A and B to a 250 mL Erlenmeyer flask using a volumetric pipette. Add 40 mL of water and heat to boiling. Trickle the standard solution without stirring until almost the end of the titration, maintaining the temperature at boiling point. Add one drop of methylene blue solution 1% and complete titration until the indicator is bleached. The time of titration should not exceed 3 min. The final titration product is around 10 mL of standard glucose solution. The result of the Fehling's solution is obtained by Eq. (11).

$$T = \frac{V \times m}{100}, \tag{11}$$

where V = volume of glucose spent in titration (mL), m = glucose mass (g).

1. *Preparation of the main sugar solution*: weigh 2 g of honey with an analytical balance in a 100 mL beaker and transfer to a 200 mL volumetric flask using distilled water.
2. *Sample preparation for titration of reducing sugars*: from the main solution (1), transfer 50 mL (mass = 0.5 g) to the 100 mL volumetric flask and complete the volume with distilled water.
3. *Sample preparation for titration of total sugars* (*total reducing sugars*): from the main solution (1), transfer 50 mL (mass = 0.5 g) to the 100 mL volumetric flask and add 25 mL of distilled water. Heat the bath solution at 64°C; add 10 mL of a distilled water solution plus p.a. HCl (8 mL of distilled water plus 2 mL of p.a. HCl), and leave in bath for 15 min. Allow the solution to cool until it reaches room temperature, and then add 2 drops of phenol-

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phthalein indicator 1% with NaOH 5 M/L solution. At this stage, a color change from light beige to pink can be seen. The volume flask is completed to 100 mL.

Titration of reducing sugars: fill the 25 mL burette with the reducing sugar solution (2) and pipette 5 mL of Fehling A and 5 mL of Fehling B into a 250 mL Erlenmeyer flask; add 40 mL of distilled water, plus five glass beads; warm until the solution boils; titrate with approximately 14 mL of the solution in the burette; wait for the solution to return to simmering temperature for 2 min; at this stage the blue staining solution contained in the Erlenmeyer flask starts to change to a purple shade; add 5 drops of methylene blue 0.2% (bluish or purple color); heat for 2 min and begin titration by adding, drop by drop, the diluted solution of honey contained in the burette until the turning point of indicator discoloration (a blue and purple color turns into a red earth color). The amount spent in titration should be noted for further calculations.

Obs.: Total titration time should not exceed 3 min.

Titration of total reducing sugars: for this titration process use the same process as above, using a solution of total reducing sugars (3). Note the volumes spent on the three replications and calculate according to Eq. (12). For calculation of sucrose, follow Eq. (13).

$$(\%) = \frac{(100 \times 100 \times 0.05)}{0.5 \times V}, \tag{12}$$

where V = volume spent in titration, 0.05 = correction factor for Fehling's solution A and B.

$$\text{Saccharose}\,(\%) = (RS - TRS) \times 0.95, \tag{13}$$

where RS = reducing sugars, TRS = total reducing sugars, 0.95 = reducing factor from total reducing sugars.

2.11. Viscosity

Viscosity and the other physicochemical properties of honey depend on many factors, including composition and temperature. One of the most important factors for viscosity is water content, as viscosity generally decreases while water content increases [42]. Studies of this trait are of great importance, as the rheological models obtained are useful for identifying the rheological properties of a fluid with practical quantities such as concentration, temperature, pH and maturation index, among others. This knowledge is essential for quality control in the intermediate control in production lines and for the design of equipment and processes [43].

Method: the principle for the determination of viscosity is the torque measuring technique, based on the resistance that the fluid exerts during rotational motion[8]. Viscosity is determined by a rotary microprocessor digital bench viscometer with thermostatic bath aid.

Turn on and reset the equipment, select the specific rotor (rotor 1 or rotor 2 spindles); turn on the water bath at 25°C; place a sufficient volume of the sample in a 250 mL beaker to cover the rotor; wait for the sample to reach the set temperature. Connect the viscometer and take the reading. The standard time to perform the reading is 1 min; the percentage of the viscometer range and the rotation per minute from the equipment vary according to each sample evaluated. After 1 min of rotation, the viscosity of each sample is read directly from the viscometer timer.

2.12. Diastase activity

As honey contains enzymes in very low quantities, this activity is the result of the joint action of diastase (α- and β-amylase), alpha-glycosidase, peroxidase, lipase, invertase, glucose oxidase, catalase and acid phosphatase. These enzymes are formed from the hypopharyngeal glands of bees and nectar sources, and are also found in low proportion in pollen grains [44]. Diastase is one of the most important enzymes, and its level in honey depends on the geographical origin and botanical source. It is an indicator of product quality [45] and its function is to hydrolyze the starch molecule. It is possibly involved in pollen digestion.

Diastase activity is closely related to the structure of the honey and can be modified by denaturing performed by overheating the honey, which seriously compromises its quality [25, 46]. In addition to shelf life and heating the product, another indicator of reduced enzyme levels are honey samples from fast nectar flows, due to the accumulation of the material processed inside the hive.

Method: the principle of the method used to evaluate the diastatic index is proposed by the AOAC [18]. This technique measures the activity of alpha-amylase in honey in the presence of starch and indirectly provides information about the quality of the honey according to the degree of digestion experienced by the starch molecule over time. To carry out this analysis, some solutions should be prepared.

Preparation of iodine stock solution: weigh 22 g of p.a. potassium iodide with an analytical balance in a 250 mL beaker and add 100 mL of distilled water for the homogenization thereof. Weigh 8.8 g of p.a. iodine in an analytical balance and add the previous solution until complete homogenization. The solution is diluted and transferred to a 1 L volumetric flask and the volume completed with distilled water.

Preparation of iodine solution 0.0007 N: weigh 4 g of p.a. potassium iodide in a 100 mL beaker using an analytical balance, dissolve the solution with 30 mL of distilled water and transfer to a 100 mL volumetric flask. Add 1 mL of stock iodine solution and fill flask with distilled water.

Preparation of starch solution: weigh 2 g of anhydrous soluble starch in a 250 mL Erlenmeyer flask using an analytical balance and dilute by adding 90 mL of distilled water. Heat the solution in a heater plate and boil gently for 3 min. Keep the solution at room temperature until it cools. Transfer the flask solution to a 100 mL volumetric flask and complete the volume with distilled water (main solution).

Standardization of the starch solution: to use the starch solution in further analysis the required volume of distilled water to be added to the solution should first be determined. This allows the standard dilution of the starch solution to be set in order to obtain an absorbance reading in the spectrophotometer range from 0.760 to 660 nm.

Label two 50 mL beakers; pipette 5 mL of solution and 10 mL of distilled water into beaker 1, and 20 mL of distilled water into beaker 2. Remove 1 mL aliquots of the solution in each beaker and transfer to another labeled beaker; add 10 mL of the iodine solution 0.0007 N. Prepare five different concentrations so that the correct volume is found. Perform a reading in a spectrophotometer set to the amount of distilled water to be added to the sample, in order to make the reading in the selected absorbance range.

Starch solution used in the analysis: label a 100 mL beaker; pipette 5 mL of main solution into the beaker, add the amount of water defined in the previous step; withdraw an aliquot of 1 mL of solution from the beaker and transfer it to another labeled beaker; add 10 mL of the standard iodine solution 0.0007 N to this beaker, and perform an absorbance reading in a spectrophotometer in the 0.760 nm range. Standardize the starch solution for every new preparation.

Weigh 10 g of honey in a 250 mL beaker using an analytical balance; add 5 mL of buffer and 20 mL of distilled water, homogenize and dissolve; transfer the sample to a 50 mL volumetric flask; add 3 mL of sodium chloride solution 0.5 M; complete the volume with distilled water; pipette 10 mL of this solution into a 250 mL beaker and place it in a water bath at 40°C, wait for 15 min; pipette 5 mL of the starch solution heated to 40°C into the honey solution; mix it and remove 1 mL aliquots to an identified beaker at intervals of 5 min, then quickly add 10 mL of the iodine solution 0.0007 N and complete the volume with distilled water.

Determine the absorbance at 660 nm in a visible spectrophotometer and record the time elapsed between the mixing of the starch solution and the addition of the honey to the iodine. Take aliquots of 1 mL every 5 min to lower the absorbance value to 0235 nm. To determine the time the absorbance took to reach this value, plot an absorbance versus time graph. The results are expressed in the Goethe scale. The diastatic index (DI) is determined according to Eq. (14):

$$DI = \frac{300}{t}, \tag{14}$$

where t = time.

2.13. Water activity (wa)

The concept of water activity has been used to evaluate the interaction of water with other food components, as water is characterized as a major component of many foods [47]. Honey has a low water activity, a parameter which determines the available water in the food and its availability for microbial metabolism, which interferes with the microbial activity in honey. This feature gives the product microbiota stability [48], resulting in quality, preservation and longer shelf life. When there is no water available in food, the water activity measurement is equal to 0.0; however, when the sample consists entirely of pure water, then water activity is equal to 1.0 [49].

Method: the AOAC [18] method is based on the measurement of the sample dew point with internal control of the sample temperature. An infrared beam focused on a small mirror determines the precise dew point of the sample. The dew point temperature is then translated into water activity. Add 7.5 mL of honey sample to a sample capsule; close the cover on the sample chamber and wait for the vapor balance; take the reading from the display.

2.14. Total phenolics

The Folin-Ciocalteu assay was designed and standardized for the quantification of total phenols by Singleton et al. [50] and adapted by Daves [51]. The system is characterized by a mixture of sodium tungstate and sodium molybdate salts in an acid medium (hydrochloric acid and phosphoric acid), which has a yellowish color. In the presence of phenolic compounds

these salts are reduced, forming complexes (molybdenum-tungsten) and producing a bluish color. The intensity of the blue tone is proportional to the number of hydroxyl or oxidizable groups of phenolic compounds. Absorption occurs at 725 nm. Phenolics determined by Folin-Ciocalteu are often expressed as Gallic acid equivalent (GAE).

Method: total phenol concentration is determined by interpolating the absorbance of the sample based on a calibration curve constructed with standard Gallic acid, with a purity of 98%.

Preparation of Gallic acid curve: dilute 0.1531 g of Gallic acid in methanol to prepare 100 mL of an initial main solution with 1500 mgGAE/L. From this concentration obtain 10 mL of diluted solution with 0.30; 180; 330; 600; 900; 1200 and 1500 mg GAE/L. Calculate concentrations of Gallic acid equivalents (mg/L) in 10 mL of solutions prepared using Eq. (15):

$$\text{GAE(mg/L)} = \left(\text{mg GA/mL from main solution} \times \text{pipetted volume}\left(\text{mL}\right)\right) \times 100, \quad (15)$$

where GAE (mg) from the main solution (mg/mL) = 1.5 mg GAE/mL.

Pipetted volume from the main solution (mL) = 0.0, 0.1, 0.2, 1.2, 2.2, 4.0, 6.0, 8.0 and 10 mL.

Adjust the volume of solutions to 10 mL using water as solvent.

Transfer 30 μL of the diluted solutions; 2.370 μL of distilled water and 150 μL of Folin-Ciocalteu reagent to test tubes protected with aluminum foil (put distilled water in the blank sample). After 2 min, add 450 μL of sodium carbonate 15%. Close the tubes and place them in a water bath with stirring in the dark at a temperature of 37°C for 30 min. Measure the absorbance in quartz cuvettes in a spectrophotometer at a wavelength of 725 nm. Plot the Gallic acid concentration (mg/L) on the abscissa (*x*-axis) and the absorbance values on the ordinate (*y*-axis). Find the coefficient of the determined R^2 value and the corresponding linear equation, as shown in **Figure 1**. Express the results in mg GAE/L.

Preparation of initial honey solution: weigh 4 g of honey and transfer it to a 10 mL volumetric flask using distilled water as the solvent, to a honey solution concentration of 0.4 g/mL. From this honey solution, transfer 30 μL to amber test tubes or tubes protected with aluminum foil (put methanol in the blank sample); add 2.370 μL of distilled water and 150 μL of Folin-Ciocalteu reagent to test tubes protected with aluminum foil (put distilled water in the white). After 2 min, add 450 μL of sodium carbonate 15%. Close the tubes and place them in a water bath with stirring in the dark at a temperature of 37°C for 30 min. Measure the absorbance in quartz cuvettes in a spectrophotometer at a wavelength of 725 nm.

Calculation of phenolic compounds: using absorbance values (*y*) and the linear equation, find the *x* value corresponding to the total phenol content in GAE/L (1000 mL); using the total phenol values in GAE/1000 mL of the main Gallic acid solution, calculate the corresponding values in 10 mL of the honey solution used (containing 0.4 g of honey/mL). From these results, calculate the concentration of total phenols in GAE/100 g of honey. Calculate the mean and standard deviation and express the results in GAE/100 g of honey ± deviation found.

2.15. Total flavonoids

Among the active principles present in nature, flavonoids are found in fruits, vegetables, seeds, flowers and bark, wine, cereals and food dyes. The aluminum chloride ($AlCl_3$) colo-

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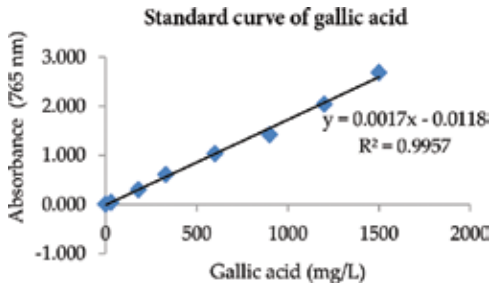

Figure 1. Standard Gallic acid curve (Gallic acid concentration × absorbance).

rimetric method is used to obtain the limits of the flavonoid spectra. Interference from other phenolic compounds is frequently present, as the Al^{3+} cations form stable complexes with free hydroxyl groups of flavonoids. This causes the extension of the conjugated system and consequently a bath chromic shift, or in other words, a shift of the absorption maxima to a longer wavelength region, allowing quantification in a spectrophotometer at 425 nm [52].

Method: total flavonoid concentration is determined by the method of Alothman et al. [53] involving the interpolation of sample absorbance based on a calibration curve constructed with standard quercetin Sigma-Aldrich™, 95% purity.

Preparation of quercetin curve: dilute 0.5263 g of quercetin in 100 mL of methanol p.a. to prepare an initial main solution of 500 mg quercetin/L. From this concentration, obtain 10 mL of diluted solutions with 2.5, 5.0, 12.5, 25.0, 37.5, 50.0, 100.0 and 150.0 mg quercetin/L. Calculate concentrations of quercetin per liter (mg/L) of diluted solutions using Eq. (16):

$$\text{Quercetin (mg/L)} = (\text{mg quercetin/mL main solution} \times \text{pipetted volume (mL)}) \times 100, \quad (16)$$

where quercetin in the main solution (mg/mL) = 5.0 mg/mL, volume of the pipetted main solution (mL) = 0.005, 0.010, 0.025, 0.050, 0.075, 0.100, 0.200 and 0.300.

Adjust the volume of solution to 10 mL using methanol as solvent.

To obtain the curve, transfer to amber color test tubes or tubes protected with aluminum foil, 250 μL of sample (put methanol in the blank sample); 1000 μL of distilled water; 75 μL $NaNO_2$ 5% in water; 600 μL of distilled water. Shake vigorously by vortexing and measure the absorbance in quartz cuvettes at 425 nm in a spectrophotometer. Plot the quercetin concentration (mg/L) on the abscissa (*x*-axis) and the absorbance values on the ordinate (*y*-axis). Find the coefficient of the determination value R^2 and the corresponding line equation (use **Figure 2** as an example). Express the results as mg quercetin equivalent/L.

Preparation of initial honey solution: weigh 4 g of honey and transfer it to a 10 mL volumetric flask using methanol as solvent for a solution with a honey concentration of 0.4 g/mL. From this solution, transfer 250 μL to amber test tubes or tubes protected with aluminum foil (put methanol in the white); 1000 μL of distilled water; 75 μL $NaNO_2$ 5% in water. After 5 min add 75 μL $AlCl_3$ 10% in water. After 6 min, add 500 μL NaOH 1 M; 600 μL of distilled water. Shake

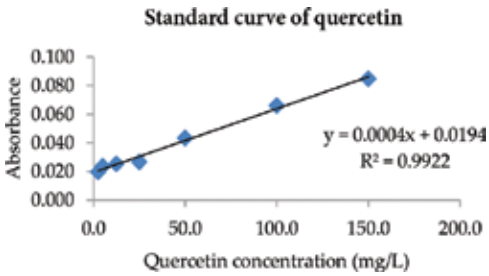

Figure 2. Standard quercetin curve (quercetin concentration × absorbance).

vigorously by vortexing and perform an absorbance reading at 425 nm.

Calculation of total flavonoid: using absorbance values (y) and the linear equation find the x value corresponding to the total flavonoid in quercetin equivalent/L. Then, multiply the values by their respective dilutions and obtain the final QE values in mg/L. Using the total flavonoid quercetin equivalent/1000 mL of the main quercetin solution, calculate the corresponding values in 10 mL of the honey solution (containing 0.4 g of honey/mL). From these results, calculate the total flavonoid concentration in quercetin equivalent/100 g of honey. Calculate the mean and standard deviation and express the results as quercetin equivalent/100 g of honey ± deviation found.

2.16. Ability to kidnap stable free radical 2,2-diphenyl-1-picrylhydrazyl—DPPH

Antioxidant activity is determined by the scavenging capacity of the free radical DPPH (2,2-diphenyl-1-picrylhydrazyl). The method involves reducing an alcoholic solution of purple DPPH radicals, which, upon receiving an electron or hydrogen radical, changes color from violet to yellow (diphenyl-picryl hydrazine),accompanied by a decrease in absorbance at the wavelength observed [54]. The greater or lesser capacity of the sample to reduce DPPH, or in other words to prevent oxidation, is evidenced by the percentage of DPPH remaining in the system [55]. This free radical, stable at room temperature, is reduced in the presence of an antioxidant molecule, yielding a yellow solution.

Preparation of the DPPH solution 0.06 mM: weigh 0.0023 g of DPPH (molecular weight = 4.32 g/L) and transfer to a 100 mL volumetric flask using methanol as solvent.

Preparation of the initial honey solution: weigh 8 g of honey and transfer to a 10 mL volumetric flask using methanol as solvent, to obtain a solution with a honey concentration equal to 800 mg/mL of the main solution. From this concentration, obtain 1.0 mL of the diluted solutions with 80.0, 120.0, 200.0, 400.0, 600.0 and 800.0 mg of honey/mL. Calculate honey concentrations per mL (mg/mL) of diluted solutions applying Eq. (17):

$$\text{Honey (mg/mL)} = (\text{mg honey/mL main solution} \times \text{pipetted volume (mL)}) \times 100 \quad (17)$$

where honey in the main solution (mg/mL) = 80 mg/mL.

Volume of the main solution pipetted (mL) = 0.10, 0.15, 0.25, 0.50, 0.75 and 1.00 mL.

Adjust the volume of solution to 1.0 mL using methanol as solvent.

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Preparation of samples: transfer 0.2 mL of samples from each dilution to amber test tubes or tubes protected with aluminum foil, and then add 3.8 mL of DPPH 0.06 mM solution. The blank 0.2 mL of the sample is mixed with 3.8 mL of methanol so that the blank of each sample is used in the final equation. The negative control is prepared by mixing 3.8 mL of the DPPH solution 0.06 mM and 0.2 mL of methanol (neat standard).

After the preparation, the mix is shaken using a vortex mixer for 15 s and allowed to stand at room temperature in the absence of light for 30 min. Sample absorbance is measured in quartz cuvettes at 515 nm in a spectrophotometer. Results are expressed as a percentage of antioxidant activity (% AA) using Eq. (18):

$$AA\ (\%) = 100 - \left[\frac{(\text{Abs sample} - \text{Abs blank})}{\text{Abs control}}\right] \times 100 \quad (18)$$

where Abs_{sample} is sample absorbance; Abs_{blank} is the absorbance of the blank control and $Abs_{control}$ is the absorbance of the negative control.

Calculation of EC_{50}: Plot the graph using the abscissa (x-axis) for concentrations of the tested honey (80.0, 120.0, 200.0, 400.0, 600.0 and 800.0) and the ordinate (y-axis) for the antioxidant activity values calculated separately for each repetition [56]. Using linear equations, compute the x values corresponding to the EC_{50} value with the y values equal to 50, which represents the minimum concentration required to reduce the antioxidant initial concentration of DPPH by 50%, represented by the curve, as the dose-response gradient is the concentration of the compound at which 50% of the effect is observed.

Calculate the mean EC_{50} value and standard error. The smaller the value, the higher is the antioxidant activity of the compounds present in the samples analyzed.

The completion of the analyses required under national and international law and those proposed in this chapter are required to determine the quality of honey for marketing, direct human consumption or use as a raw material for the food, cosmetics and pharmaceutical industries.

The results of sensory, physicochemical and functional properties analysis allows us to evaluate if the product meets established standards and demonstrates the features expected from good quality honey.

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Chapter 10

Antimicrobial Activity of Honey

Piotr Szweda

Additional information is available at the end of the chapter

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Abstract

Honey has had a valued place in traditional medicine for centuries. It was used to overcome liver, cardiovascular and gastrointestinal problems and for treatment of some types of infectious disease. Particularly, good results were achieved in the case of application of this product for therapy of infected, difficult to heal wounds. The high health-promoting properties of honey have been recently confirmed in many research investigations. The antimicrobial activity of this product is highly complex. Generation of hydrogen peroxide, bee defensin-1, high osmolarity and low value of pH seems to be crucial for its antimicrobial potential. Considering honey as a therapeutic, antimicrobial agent special attention deserves Manuka honey. Its high antimicrobial activity is caused by high concentration of 1,2-dicarbonyl compound methylglyoxal. Some authors also suggest that other phytochemicals, especially phenolic compounds, are important antibacterial ingredients of honey. The results of many in vitro but also in vivo studies confirm high antimicrobial potential of honey against some important human and veterinary pathogens: *Staphylococcus aureus, Helicobacter pylori, Mycobacterium tuberculosis, Pseudomonas aeruginosa* and *Escherichia coli*. We do not have doubts that honey, but also other bee products, especially propolis, is promising antimicrobial agents and possibilities of their application in clinical medicine deserve consideration.

Keywords: honey, glucose oxidase, bee defensin-1, polyphenols, antimicrobial activity, infectious diseases, staphylococci

1. Introduction

1.1. Honey: a beneficial food product

Due to its unique taste, nutritional value and health-promoting properties, honey has a valued place in the human diet. Sugars, mainly fructose and glucose, and minor amounts of oligosaccharides account for about 80% of its weight. As a consequence, it is an easily digestible and high energetic food product. Consumption of 100 g of honey provides the body

with about 320 kcal. However, the health-promoting properties of this product come mainly from the presence of other than sugar components: enzymes, peptides, free amino acids, vitamins, organic acids, flavonoids, phenolic acids and other phytochemicals and minerals [1]. The beneficial effects of eating honey have been confirmed by centuries of observations. Consequently, honey has become one of the major therapeutic agents of traditional medicine. Depending of botanical source, different types of honey are proposed for prophylaxis and treatment of different health problems. According to polish traditional medicine [2],

- rapeseed honey (produced from *Brassica napus* L.) soothes liver disease, and it is also recommended in therapy of diseases of the cardiovascular system and kidneys;
- honey produced from acacia (*Robinia pseudoacacia* L.) nectar is especially recommended for diabetics, and it also helps to alleviate digestive disorders and gastrointestinal diseases;
- heather (*Calluna vulgaris* L.) honey is used for treatment prostate and liver and biliary system diseases;
- many benefits come from consumption of buckwheat (*Fagopyrum esculentum* Moench) honey; it relieves the symptoms of hypertension and atherosclerosis and promotes regeneration of bone tissue. It is also recommended to diabetics and for treatment of inflammatory conditions of the kidney, urinary tract and joints;
- good results in the treatment of depression an neuroses have been obtained by the use of honey sourced from buckwheat, linden tree (*Tilia* spp.) and also some multifloral and honeydew honeys;
- diaphoretic and antipyretic effects have been confirmed for raspberry (*Rubus idaeus* L.) and linden tree honeys; as a consequence, these honeys are popular in treatment of influenza and bacterial infections.

In particular, interesting and important issue is antimicrobial activity of honey. In fact, it is the only food product that without any technological processing, nor addition of preservatives, can be stored for a long period of time—even several years, without any negative symptoms. Interestingly, the honey is not a sterile product (**Figure 1**).

It contains some microorganisms, mostly bacteria yeast and molds. However, the specific environment of this product, high osmotic pressure (high concentration of sugars) and high acidity (low value of pH) prevents the development of microorganisms [3]. As a consequence, only some groups of bacteria and fungi are able to exist within environment of honey and the population of microorganisms is stable during storage. Moreover, it has been shown that some of bacteria that are present in the honey produce antimicrobial agents, bacteriocins, which can protect the product against development of other microorganisms and are be beneficial for consumers' health [4]. Antimicrobial potential of honey has been successfully used in folk medicine, with a particularly good result in the case of therapy of infected, difficult to heal wounds [5]. Since the introduction to the clinical practice sulfonamides and next antibiotics treatment of infectious diseases with natural products, including honey was minimized. Due to observed recently rapid increase in isolations of strains resistant to a plethora of antibiotics, the possibility of using herbs, honeybee products and other natural products for the treatment of infection is again seriously considered. The aim of preparing this chapter

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is presenting perspectives not only limitations of application honey for treatment but also prophylaxis of diseases caused by microorganisms, especially bacteria.

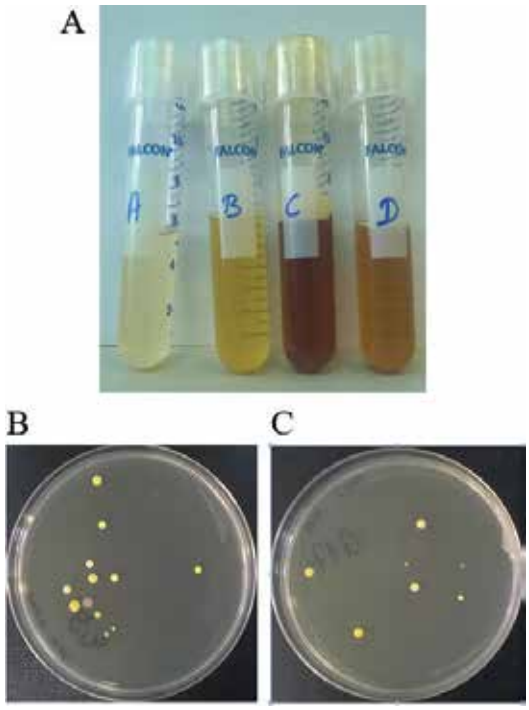

Figure 1. (A) Solutions of investigated honeys (25%, v/v): A, rapeseed honey (*Brassica napus* L.); B, multifloral honey; C, buckwheat honey (*Fagopyrum esculentum* Moench); D, Manuka honey (*Leptospermum scoparium*). Some characteristic differences in the color of the solutions are visible. (B) Result of growing of 50 μl of solutions of honeys A and B on LA agar. In both cases, several colonies were obtained, which confirm contamination of investigated honeys with some bacteria.

2. Mechanism of antimicrobial activity of honey

The antimicrobial activity of honey is highly complex and still remains not fully recognized. To date, it has been established that several components of this product play a crucial role for its antimicrobial properties [6–11]:

- high concentration of sugars (about 80% of weight of this product) eliminates microorganisms, mainly bacteria that are sensitive to high osmotic pressure and inhibit the development of more osmotolerant microorganisms;
- low pH value—high concentration of organic acids (e.g., gluconic acid). The pH of most honey types is in the range from 3.4 to 6.1, which in combination with high osmotic pressure eliminates or enables the development of most microorganisms;
- bee defensin-1, it is a peptide secreted by the honeybee hypopharyngeal glands. As a component of royal jelly (it is also called royalysin), it probably plays a key role in the health of bee larvae. It exhibits activity against Gram-positive bacteria, including *Bacillus subtilis*,

Staphylococcus aureus and *Paenibacillus larvae* (etiological agent of important bee larval diseases American foulbrood). While high concentration of sugars and low pH are universal antibacterial factors of all honeys, strong differences have been noticed in the case of amount of this peptide in different honey and royal jelly samples. The bees also produce at least three other antibacterial peptides as important components of their innate immune system. However, to date, they have not been detected in honey;

- glucose oxidase—the enzyme, oxidoreductase that catalyzes the oxidation of glucose to gluconic acid. The side product of this reaction, hydrogen peroxide (H_2O_2), is a strong antimicrobial agent. The detailed mechanism of this reaction is presented below (**Figure 2**). The enzyme is produced in honeybees' salivary glands and introduced to the collected nectar. It protects the ripening honey against the development of pathogenic microorganisms. Interestingly, the enzyme is present but not active in the mature honey; this product is sufficiently protected with high osmotic pressure and low acidity. When the honey is diluted, the enzyme regains activity, which is extremely important for honeybees and especially their larvae health. Honey is the most important component of honeybees' diet; however, before consumption, it is diluted in water. The generated by the enzyme H_2O_2 is a major antimicrobial defense factor for this diluted honey. Its production is also crucial for antimicrobial potential of honey used for treatment of skin and soft tissue infections, infected wounds or eradication pathogenic bacteria located within upper respiratory tract or *Helicobacter pylori* located in human stomach.

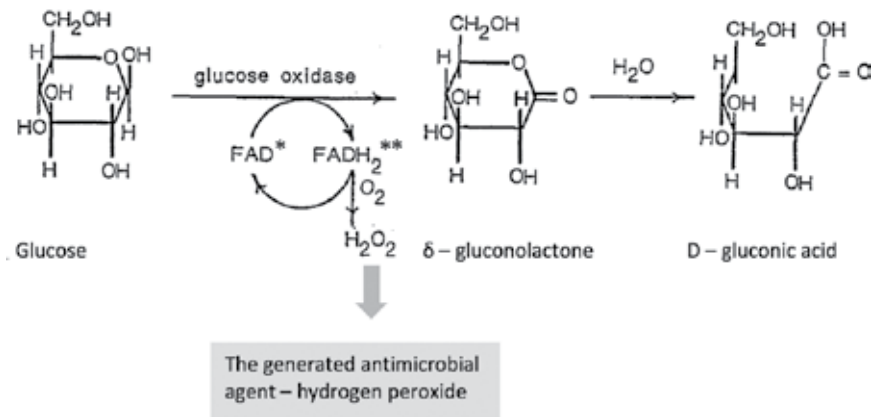

Figure 2. The reaction calalyzed by glucose oxidase—generation of hydrogen peroxide.

The unique antimicrobial properties have been identified for honey produced from the Manuka bush (*Leptospermum scoparium*) indigenous to New Zealand and Australia. In contrast to majority of other nectar and honeydew honeys, the crucial factor responsible for the bactericidal activity of this product is high concentration of 1,2-dicarbonyl compound methylglyoxal (MGO) [10–12]. Some authors also suggest that other phytochemicals, especially phenolic compounds, are important antibacterial ingredients of honey. Evident differences in activity of honeys produced from different botanical sources seem to support this hypothesis [13–15]. However, the observed differences could be also caused by differences in activity or

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concentration of glucose oxidase or concentration of defensin-1, which has not been investigated to date. The presence of phenolic compounds in honey has been confirmed in several independent studies. The results of these studies also revealed that concentrations of individual constituents are too low to substantially contribute to the antimicrobial activity of the product. It is possible, however, that combination of different ingredients, for example, phenolic compounds, might significantly contribute the activity of honey [6, 11, 16]. Quite satisfactory activity of composition of phenolic compounds extracted from several Malysian and Polish honeys was observed, respectively, by Aljadi and Yusoff [17] and Mazol and coworkers [18]. Interesting results in this area have been also presented by Mundo and coworkers (2004) [15], who observed non-peroxide activity against *Bacillus stearothermopnilus* in most of 27 honey samples diluted in water containing catalase (the enzyme degrading hydrogen peroxide). In contrast to *Bacillus*, the neutralization of H_2O_2 with catalase resulted in loss of activity against *S. aureus* in the case of all tested honey samples except of two samples of horsemint honeys. This result could suggest the presence of some nonproteinaceous components in these honeys, which were responsible for inhibition of growth of *Bacillus* in the suspensions of honey not containing hydrogen peroxide [15]. The presence of antimicrobial components (combination of cationic and noncationic but not identified substances) other than methylglyoxal, glucose oxidase and defensin-1 in Manuka honey was confirmed in the studies of Kwakman and coworkers [10, 11]. These authors also investigated that the other honeys, assigned as RS (Revamil—medical grade honey) and completely opposite results, were obtained. In the case of this product neutralization of H_2O_2, MGO, defensin-1 and subsequent titration of honey to neutral pH resulted in complete loss of antimicrobial activity [10, 11]. On the basis of current state of knowledge, it rather should be assumed that phytochemicals, except of methylglyoxal, are not crucial for antimicrobial potential of most honeys. However, in the case of honeys produced from some botanical sources, they probably substantially support the primary factors: pH, high osmolarity and defensin-1 (in the case of undiluted honey) and hydrogen peroxide in the case of diluted product. Thus, the contribution of phytochemicals to the antimicrobial activity of honey remains unclear and needs to be investigated.

The investigation carried out by Lee and coworkers [4] revealed that honey is a promising source of bacteriocinogenic bacteria strains. The mentioned authors analyzed two Manuka honey samples from New Zealand and six domestic honeys from the United States of America. The 2217 isolates out of 2398 strains (92.5%) exhibited activity at least against one of the tested microorganisms. Among them, 1655 exhibited activity against *Listeria monocytogenes* and 1605 inhibited the growth of another important human and veterinary pathogen *S. aureus* [4]. Beside of that, at the moment, it is rather difficult to classify bacteriocins as the next important antibacterial component of honey. To date, only the strains producing these peptides have been isolated from honey, the presence of bacteriocins within the product has not yet been confirmed.

3. Determination of antimicrobial activity of honey

The in vitro antimicrobial activity of most agents is usually estimated with two methods: an agar diffusion assay and a serial dilution method in microtiter plates. Both these methods have been also used for determination of antimicrobial potential of honey. The agar diffusion

assay is based on the measurement of size of growth inhibition zone around the place of loading a sample of honey (usually well, cut with a cork borer in the agar). The assay is easy and quick in performance. Unfortunately, it has several important limitations [11]:

- high viscosity of honey and problems with loading of defined volume of the product sample to the wells in the agar. It is especially problematic when the honey is crystallized;
- problems with diffusion of active components (defensin-1 and especially glucose oxidase characterize with high molecular weight) through the agar matrix. As a result, the diameters of observed growth inhibition zones are relatively low. The honeys with evidentially different activities established with other methods give similar results in agar diffusion assay (not large differences in the diameters of growth inhibition zones are observed—based on results of own studies, **Figure 3**);

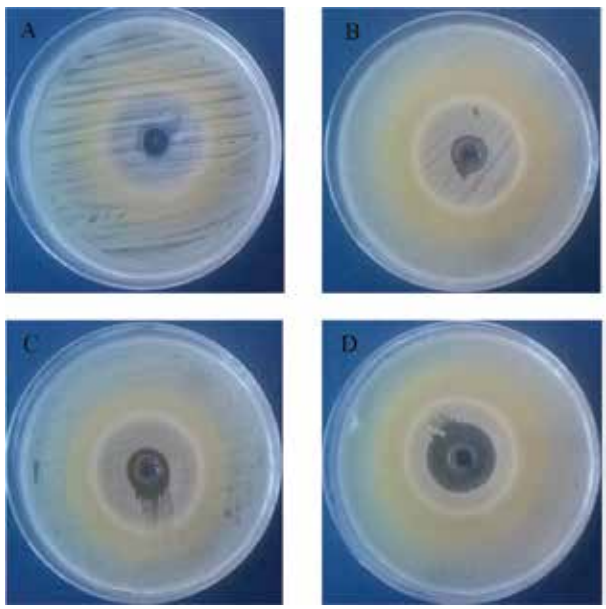

Figure 3. Results of agar diffusion assay of activity of four selected honeys: (A) rapeseed honey (*Brassica napus* L.); (B) multifloral honey; (C) buckwheat honey (*Fagopyrum esculentum* Moench); (D) Manuka honey (*Leptospermum scoparium*). Definitely the highest activity was observed in the case of Manuka honey—picture D. Some characteristic halo zones are present in all pictures. 100 μl of 50% (v/v) was loaded to the wells in the agar.

- low reproducibility—it is difficult to get similar results (diameter of growth inhibition zone) in several independent experiments;
- low discriminatory power—consequence of relatively low sizes of observed growth inhibition zones. It is also difficult to compare the obtained results with the results of other authors;
- lack of possibilities to distinguish bacteriostatic and bactericidal activity;
- problems with interpretation of obtained results. Usually except of clear, growth inhibition zones at least one halo zone can be observed (**Figure 3**). In this halo zone, the colonies of

growing bacteria characterize with different (lower) diameter, and some changes of color of agar can be noticed, which is difficult in interpretation (**Figure 3**). In our opinion, the presence of these halo zones is a consequence of influence of low molecular components of honey on the growth of microbial cells.

The problems with high viscosity can be, at least partly, omitted by using honey dissolved in sterile water (e.g., 50%, w/w), as it has been proposed by several authors [15]. However, usually it does not solve other discussed above problems.

Based on our experience, we would rather recommend a serial dilution method for investigation of antimicrobial potential of honey [13]. This method allows quantitative determination of both bacteriostatic and bactericidal activity of tested honey samples, **Figures 4** and **5**. The bacteriostatic activity is characterized with MIC (*Minimal Inhibitory Concentration*—the lowest concentration of honey that inhibits the growth of tested strain of microorganisms) parameter, while bactericidal activity is characterized with MBC (*Minimal Bactericidal Concentration*—the lowest concentration of an antibacterial agent required to kill a particular bacterium) parameter.

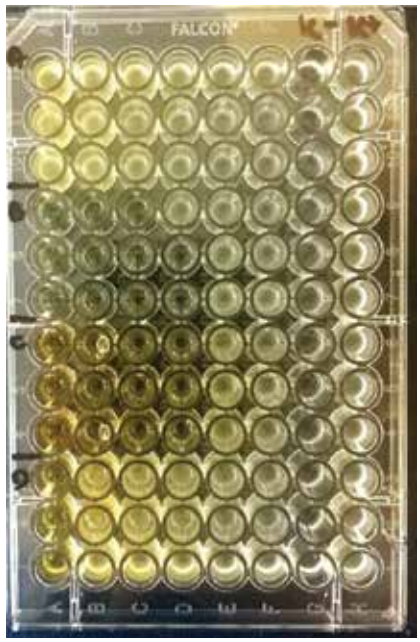

Figure 4. The results of determination of antistaphylococcal activity of four tested honeys: (A) rapeseed honey (*Brassica napus* L.)—rows 1–3; (B) multifloral honey—rows 4–6; (C) buckwheat honey (*Fagopyrum esculentum* Moench)—rows 7–9; (D) Manuka honey (*Leptospermum scoparium*)—rows 10–12. The concentrations of honeys in the wells of following columns were as follows: 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39% (v/v). The wells of column number 7 contained only growing medium (Mueller Hinton BrothII cation adjusted) neither honey nor cells of bacteria were present in these wells—negative control. The wells of column 8 did not contain honey, and they were used as a positive control of growth of bacteria in the medium not containing any antimicrobial agent. No activity was observed in the case of rapeseed honey. The reference strain of bacteria *S. aureus* PCM1051 was able to grow in all wells of rows 1–3. The MIC value for multifloral honey (the lowest concentration of honey, which caused visible inhibition of growth of *S. aureus* strain), was 3.12% in row 4 and 1.56% in rows 5 and 6. The MIC value for buckwheat honey in all three tested rows was 1.56%, and the constant value of MIC for Manuka honey, 12.5%, was observed in the rows 10–12.

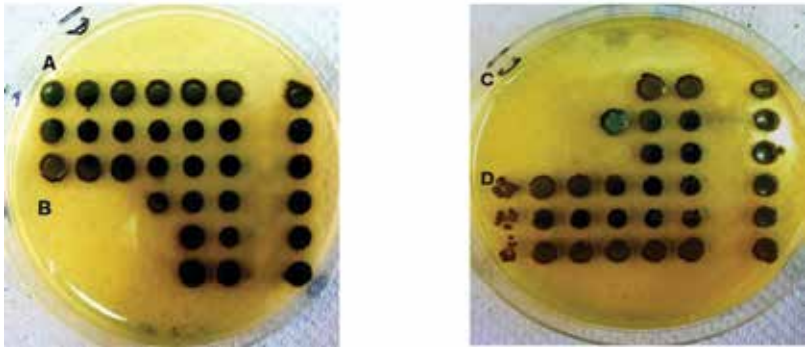

Figure 5. The results of determination of minimal bactericidal concentrations of tested honeys against *S. aureus*. The assay (carried out according to the procedure presented in **Figure 6**) confirmed the lack of activity of rapeseed honey. The MBC values of multifloral honey were exactly the same as MIC values for this product. In the case of buckwheat honey, MBC and MIC values were the same in the rows 7 and 9, and in the case of row 8, the MBC was twice of the MIC value, 3.12 and 1.56% (v/v), respectively. Interestingly, no bactericidal activity was observed in the case of Manuka honey. However, the intensity of growth of bacteria transferred from the wells with the highest concentration of this honey (12.5%) was evidentially inhibited in comparison with samples presenting wells containing lower concentrations of this product. Despite the fact that the same product was tested in triplicate, in the case of determination of activity of multifloral and buckwheat honeys, some differences in the obtained MIC and MBC values were observed for different rows (e.g., MBC value for buckwheat honey was 1.56% in the case of rows 7 and 9, and in the case of row 8, the bactericidal effect was achieved at the concentration of 3.12%). However, the observed differences of determined values of the parameters of interest for particular honey were not larger than two times, which is acceptable in these assays.

The detailed description of procedure of performance of this assay as well as determination of both parameters MIC and MBC has been presented in **Figure 6**. The most problematic step of this assay is preparing the output solution of honey; in our laboratory, it is usually 25% (v/v). Because of high viscosity of honey, the determination of volume of the product used for preparing the solution has to be done extremely carefully. Other way, it can be a source of significant measurement errors. There are also several other advantages of serial dilution method in comparison with agar diffusion assay. The dilution assay gives more reproducible results, which are easy in interpretation. It also characterizes with much better discriminatory power, for example, results presented in **Figures 4** and **5**.

Slightly modified serial dilution method can be also used for determination of antibiofilm activity of honey. Minimum biofilm eradication concentration (MBEC—the lowest concentration of an antibacterial agent required to eradication of biofilm formed by a particular bacterium) of honey is determined in this assay. In general, the assay is performed identically as in the case of determination of MIC or MBC parameters. However, in the first step, the bacterial biofilm is grown in the wells of titration plates.

Preparing this chapter, we performed some assays of activity of four selected honeys: A, rapeseed; B, multifloral; C, buckwheat; D, Manuka honey against *S. aureus* PCM2054 reference strain. As it is presented in **Figures 3** and **4**, some important differences in the results of these two assays have been obtained. In the case of agar diffusion assay, definitely the highest activity was observed for Manuka honey, while in the case of dilution method, buckwheat

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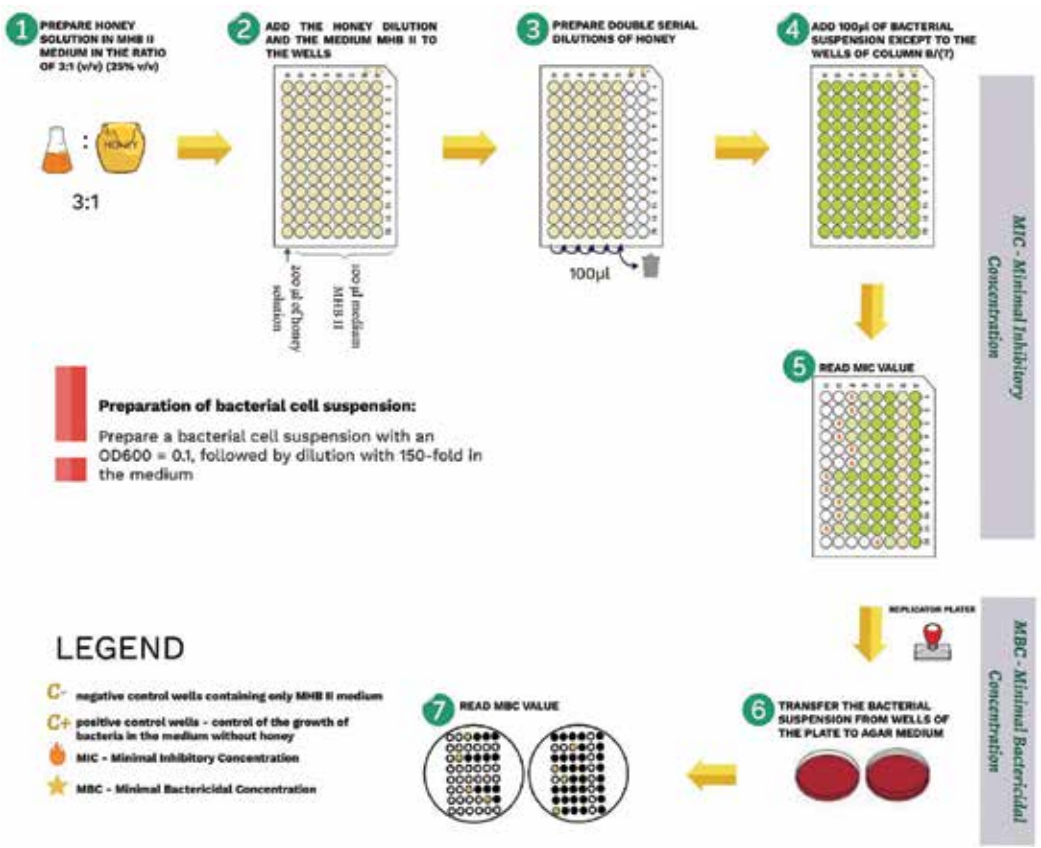

Figure 6. The procedure of performance of serial dilution method. The MHBII medium used for honey dilution should be prepared with using of only 75% of water volume recommended for this medium. The required volume of medium will be obtained in the consequence of adding of honey. MHBII medium used for serial dilution of honey and preparing of suspension of bacterial cells should be prepared according to manufacturer's procedure (with using recommended volume of water).

and multifloral exhibited much better activity in comparison with the honey produced in New Zealand. Rapeseed honey in both assays was classified as non-active. These results are in agreement with our previous observations [13]. Our previous research revealed also that activity of polish honeys is hydrogen peroxidase dependent [13]. Thus, the relatively low activity of buckwheat and multifloral honey in the case of agar diffusion method was probably a consequence of difficulties of migration glucose oxidase through the agar. Activity of Manuka honey comes mainly from high content of MGO, which is a low molecular weight component that can easily migrate through the agar generating a large growth inhibition zone. It is also worth to notice that MBC and MIC values for buckwheat and multifloral honeys are the same (1.56%, v/v). The MBC value for Manuka honey could not be determined in the tested range of concentrations; however, evident inhibition of growth of *S. aureus* in the wells containing 12.5% (v/v) of the honey is visible in both: titration plate as well as on the Petri dishes with Baird-Parker agar.

4. Antimicrobial activity of honey: in vitro and in vivo studies

High antimicrobial potential of honey has been confirmed in many in vitro tests, but also in vivo studies. Some important differences in the activity of honey produced from different botanical sources have been revealed. In general, molds and yeasts are less sensitive to the activity of this product, as high concentration as 30–50% is necessary to inhibit the growth of these groups of microorganisms [11]. Much higher activity has been observed in the case of bacteria, especially sensitive are Gram-positive bacteria. Taking into account mechanisms of activity, methods of application and dose of the product necessary for effective elimination of bacteria only some specific types of diseases could be treated with honey, for example, infected wounds, skin and soft tissue infections, infections located within upper respiratory tract, mucosa of digestive tract, vaginal mucosa and some specific disease, for example, stomach ulcers caused by *Helicobacter pylori*. This limitation was the main criterion for selection of bacterial species for presented below description concerning the results of research of antimicrobial activity of honey and possibilities of its application in clinical practice or prophylaxis of some disease.

4.1. Antistaphylococcal activity of honey

High, in vitro, antistaphylococcal activity of honey has been confirmed by many researchers—for details please see our previous review [19]. In fact, staphylococci belong to the most sensitive bacteria to the components of this product [19]. The growth of staphylococci is inhibited by proteinaceous components—defensin-1 and generated by glucose oxidase H_2O_2, as well as by other antimicrobial ingredients: mainly polyphenols and methylglyoxal in the case of Manuka honey. Our previous research revealed high antistaphylococcal (against *S. aureus* PCM 2051—reference strain) activity of polish honeys produced from cornflower (*Centaurea cyanus* L.), buckwheat (*Fagopyrum esculentum* Moench) and thyme (*Thymus vulgaris* L.) with MIC values of 3.12 or 6.25% (v/v); some differences of activity of different samples of honey obtained from the same botanical sources were observed [13]. The obtained results (the ranges of effective concentrations) are in agreement with the results presented by other authors who investigated honeys sourced from different geographical locations, for example, from Greece [14] or Iran [20]. High antistaphylococcal activity of honey has been also confirmed for MRSA (*Methicillin-Resistant Staphylococcus aureus*) clinical isolates. Effective inhibition of growth of MRSA isolates has been revealed in the case of Chilean honey obtained from Ulmo tree [21], Malaysian melaleuca honey [22], some Thai honeys, especially from longan flower [23], Finland [24], Ethiopia [25] and several other geographical regions. Honey is also effective in eradication of staphylococcal biofilm. Lu and coworkers [26] revealed that New Zealand Manuka-type honeys, at the concentrations they can be applied in wound dressings, are highly active in both preventing *S. aureus* biofilm formation and in their eradication and do not result in bacteria becoming resistant [26]. High efficiency in elimination of bacterial biofilm confirmed also for honeys whose activity depends mainly from hydrogen peroxide generation, for example, "Medihoney"—therapeutic honey and Norwegian Forest Honey [27]. Staphylococci are often isolated from skin and soft tissue infections; they are also important etiological factor of wound infections. The group of Blaser achieved a full

healing in seven consecutive patients whose wounds were either infected or colonized with methicillin-resistant *S. aureus*. Antiseptics and antibiotics had previously failed to irradiate the clinical signs of infection [28]. Interesting results were also presented by Al-Waili [29] who used honey for treatment surgical wounds made on the dorsum of mice infected with different species of bacteria. It was found that local application of raw honey on infected wounds reduced redness, swelling, time for complete resolution of lesion and time for eradication of bacterial infection due to *S. aureus* or *Klebsiella* sp. Its potency was comparable to that of local antibiotics [29]. Because of their promising properties, the wound dressing materials containing honey (mostly Manuka honey) are already commercially available and gain popularity in treatment difficult to heal infected wounds.

4.2. Activity against *Helicobacter pylori*

In vitro anti-*H. pylori* activity of honey has been confirmed by several research groups. Using agar diffusion assay, Nzeako and Al-Namaani [30] investigated activity of eight samples of honey (four from Germany, one from Switzerland, one from Iran and two from Oman). All of them effectively inhibited the growth of *H. pylori*. The size of growth inhibition zones produced by the samples of 100 μl of undiluted honey varied from 15 mm for Blossom bee honey (Switzerland) to 29 mm for Al-Nada clove honey (Oman) [30]. Interesting results of in vitro studies of anti-*H. pylori* activity of three locally produced honeys from different regions in South Africa were presented by Manyi-Loh and coworkers [31]. The authors revealed high activity of honey but also extracts of organic, nonproteinaceous components of these products [31]. Al Somal and colleagues [32] revealed much better anti-*H. pylori* activity of Manuka honey in comparison with peroxide-dependent honey. All five isolates tested by the authors were sensitive to a 20% (v/v) solution of Manuka honey in an agar well diffusion assay, but none showed sensitivity to a 40% solution of a honey sample in which the antibacterial activity was due primarily to its content of hydrogen peroxide [32]. The observations presented by the groups of Manyi-Loh et al. [31] are especially important from the point of view of specific conditions in stomach. High concentration of HCl and low value of pH certainly affects the activity of enzymes that are present in consumed food, including glucose oxidase, which generates hydrogen peroxide and is crucial for antimicrobial activity of most types of honeys. Thus, the presence of other than H_2O_2 antimicrobial components in honey is very important for possibilities of its effective application for prophylaxis and therapy of in vivo *H. pylori* infections. Recently, Sahin [33] revealed that phenolic components of chestnut and oak honeys effectively inhibited activity of two enzymes: urease and xanthine oxidase, which are important virulence factors of *H. pylori*. These results importantly confirm that regular consumption of honey (especially the products rich in polyphenols) could prevent gastric ulcers deriving from *H. pylori* [33]. Moreover, analyzing the group of 150 dyspeptic patients, Boyanova and colleagues (2015) [34] revealed that consumption of honey at least 1 day weekly significantly reduces the risk of development of infection with *H. pylori* [34]. The in vitro susceptibility of *H. pylori* to honey is well documented. In our opinion, more studies aiming in evaluation of in vivo effects of regular consumption of honey for development of *H. pylori* infection within stomach are necessary. These researches should concentrate on selection of type of honeys (probably characterized with high content of polyphenols and/or MGO), especially effective in eradication this bacterium from the tissue.

4.3. Activity against *Mycobacterium tuberculosis*

M. tuberculosis, being the leading member of the MTB complex (*Mycobacterium tuberculosis complex*), is the main cause of tuberculosis worldwide. Over the recent past years, resistance against antituberculous drugs has emerged rapidly, resulting in MDR (*Multi Drug Resistant*) strains. In vitro activity of Beri honey (from Pakistan) was tested against 21 clinical isolates of MDR-MTB by Hannan and coworkers (2014) [35]. The obtained results clearly demonstrate that Pakistani Beri honey exhibits significant antimycobacterial potential, and three (14%) of the isolates were susceptible at 1% (v/v) honey, while at 2% (v/v) of honey, 18 (86%) isolates were found to be susceptible. All the 21 isolates ($n = 21$) were susceptible at 3% (v/v) of honey [35]. Honey was also proposed for treatment tuberculosis by Avicenna, a known ancient Persian philosopher and physician. At the beginning of twenty-first century, this hypothesis was evaluated by the researchers from Shiraz University of Medical Sciences in Iran [36]. It was demonstrated that the growth of mycobacteria was inhibited by adding 10% honey to the growing media (Lowenstein-Jenson media and L-J media were used). *Mycobacteria* did not grow in culture media containing 10 and 20% honey, while it grew in culture media containing 5, 2.5 and 1% honey. Thus, the obtained results of in vitro tests are quite optimistic [36]. However, future research of in vivo activity of honey against *Mycobacteria* located within the lung tissue would be necessary for fully evaluation of its usefulness in the treatment of tuberculosis. According to the best of our knowledge to date, such studies have not been conducted.

4.4. Activity against Gram-negative bacteria: *P. aeruginosa* and *E. coli*

The most carried out to date studies revealed that Gram-negative bacteria are a bit less sensitive to the activity of honey in comparison with Gram-positive bacteria. This situation was also observed in the research carried out in our group. The collection of over 30 Polish monofloral honeys was tested, and definitely most of them were less active against *P. aeruginosa* and especially *E. coli* reference strains in comparison with *S. aureus* PCM2051. However, the activity of most of honeys against these pathogens was on satisfactory level, with MIC values in the range of concentrations from 6.25 to 25% (v/v) and from 12.5 to 25% (v/v), respectively [13]. Honey effectively eradicates biofilm formed by *P. aeruginosa* [37]. Activity of this product against this bacterium has been also confirmed in some in vivo studies. The stingless bee honey has been successfully used for treatment of *P. aeruginosa* infected conjunctivitis in Hartley guine pigs [38]. The investigation carried out by the group of Khoo (2010) revealed that Tualang honey-treated rats demonstrated a reduction in bacterial growth in *P. aeruginosa* inoculated wounds [39]. *P. aeruginosa* belongs to the important etiological factors of wound infections. Thus, activity of many potential wound dressing materials containing honey against this bacterium has been carried out. Most of them confirm high therapeutic antimicrobial potential of honey.

5. Antimicrobial activity of other honeybee products

In addition, honeybees also produce propolis, wax, pollen, bee bread and royal jelly. All these products exhibit some antibacterial activity. However, from the point of view of possibilities

of their application for prophylaxis or treatment of infections, definitely the most promising is propolis. It is a resinous substance produced from plants' buds and exudates, modified by addition of honeybees' salivary secretions and wax. It is a product of a very complex chemical composition, which depends on many factors; in particular, important are geographical location and plant base, which is available for honeybees when collecting their products. Some of its ingredients, mainly polyphenols and flavonoids, exhibit high antimicrobial activity. As a consequence, it is used by honeybees as a hive disinfectant [40]. Ethanolic extracts of propolis exhibit high activity against wide spectrum of human and veterinary pathogenic microorganisms. The investigation carried out in our group revealed promising activity of Polish propolis against clinical isolates of azole-resistant yeasts of the genus *Candida*. In total susceptibility of 44 strains [*C. albicans* (n = 20), *C. glabrata* (n = 14) and *C. krusei* (n = 10)] were tested, and in the case of one sample of propolis, the MFC (*Minimal Fungicidal Concentration*) values were in range from 0.156 to 1.25% (v/v) [41]. Many studies also revealed high activity of propolis against Gram-positive bacteria, including as dangerous pathogens as *S. aureus, S. epidermidis, Listeria monocytogenes* [42], *Bacillus subtilis* and *B. cereus* [43]. It has been also confirmed that ethanolic extracts of propolis enhance activity of some antibiotics against staphylococci [44]. Some important Gram-negative bacteria also exhibit sensitivity to the components of propolis. However, the research of propolis from different regions of the world is consistent and indicates that higher concentrations are necessary for elimination of *E. coli* or *P. aeruginosa* in comparison with Gram-positive bacteria [45–47]. Propolis belongs to the most popular products used for treatment infections in traditional medicine. During last several decades, its high antimicrobial potential has been confirmed with a large number of scientific publications. We have no doubts that possibilities of application of this product in clinical medicine deserve consideration.

6. Conclusions

Honey produced from some botanical sources exhibits high antimicrobial activity. Possibilities of application of this product for treatment infections in clinical practice should be the subject of intensive investigations in the near future. Except of high activity, the most important advantages of this product are as follows:

- lack of side effects for patients (important drawback of antibiotics);
- low costs of therapies;
- low possibility of development of resistant strains—the cells of pathogens are simultaneously affected with several factors, for example, hydrogen peroxide, bee defensin-1, methylglyoxal or other phytochemicals;
- the honey provides the body of the patient many health-promoting components, for example, antioxidants, microelements, trace elements and vitamins.

However, it has to be notice that several important problems would have to be solved for more common application of honey for treatment infections.

- only the honey classified as medical grade—with confirmed antibacterial activity, free of pathogenic microorganisms and toxic components could be used in medical applications;
- each batch of raw material (honey) would have to be tested for its biological activity;
- the method of sterilization, safe for proteinaceous antibacterial components of honey (glucose oxidase, bee defensin-1), would have to be developed (gamma-irradiation sterilization seems to be promising [48]);
- much more studies are necessary to check the in vivo effects of treatment of infections;
- one method of determination of antimicrobial activity of honey should be recommended, as a consequence comparison of activity of the product tested in different laboratories would be easier (based on our experience, we would recommend serial dilution method for this purpose).

Summarizing we have no doubt that honey is an interesting and promising alternative to classical antibiotics and should be more seriously considered as therapeutic agents.

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Chapter 11

Microorganisms in Honey

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Additional information is available at the end of the chapter

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Abstract

Honey is a product with low water activity because of the great amount of sugars (fructose and glucose), and also it has antimicrobial compounds derived from flowers or because of its transformation process in the beehive. Despite all the honey microorganism barriers, some species of microorganisms are able to survive and may cause damage to honeybees or consumers. Techniques of pathogenic microorganism identification by DNA using PCR are recommended and required for sanitary and customs control. It is important to know the diversity of contaminating microorganisms in honey, especially due to disseminate pathogenic microorganisms in the international traded marketing. In contrast, beneficial microorganisms such as yeasts can remain latently in this product waiting for the moment in which the environment is suitable for their development. Among the beneficial bacteria found in honeybee products, we can mention some lactic acid bacteria that act as prebiotics when ingested. The microorganisms in the digestive tract of honeybees are important for their health. Thus, we present the knowledge of microbiota associated with honey from honeybees and stingless bees (*Hymenoptera, Apidae*) and the techniques available for the detection of microorganisms in honey.

Keywords: microbiota, prebiotics, pathogenic microorganisms, yeast, bacteria

1. Introduction

Honey is used as a therapeutic product since ancient times. Its properties are chemically evidenced by its composition. Among features that make this product effective against microorganisms, we can quote high osmotic pressure by low water activity (average 17.2%); low pH because of the presence of organic acids, mainly gluconic acid (average 3.9); the presence of hydrogen peroxide generated by action of enzyme glucose oxidase; low protein content;

low redox potential due to the presence of reducing sugars; and chemical agents present as lysozyme, phenolic acids, pinocembrin, terpenes, benzyl alcohol, and volatile substances [1, 2].

High osmotic pressure results from its composition: 85–95% of sugar, of which it has 28–31% of glucose, 22–38% of fructose, 1–4% of sucrose, and 1–9% maltose [3]. Isomaltose and some oligosaccharides are also present in honey and vary according to flowering, climate, and local production [4, 5]. As honey is a product developed from changes in nectar, the bees incorporate the glucose oxidase enzyme that converts glucose into hydrogen peroxide and gluconic acid; this compound is indeed important for the taste of products as well as their bioactivity [5, 6]. The presence of acids and other chemicals varies with the composition of the transformed nectar; for this reason, some honeys have higher antimicrobial activity with respect to other different blossoming [7].

About these conditions, few microorganisms have the capacity to develop or remain in honey. These microorganisms are derived from primary or secondary sources of contamination. The primary sources are related to digestive tract of honeybees, which have natural microorganisms and sources of material collection such as nectar, pollen and propolis, air, flowers, and the environment inside the beehive, while the secondary sources are incorporation of honey microorganisms postharvest, processing plants, and appliances [5].

2. Human pathogenic microorganisms found in honey

Due to characteristics cited above, only pathogenic bacteria capable of sporulation have the ability to keep in honey, but they have no reproductive capacity or vegetative cells. Fungi and yeasts are able to maintain their vegetative form [2].

Fungal growth is followed by the production of mycotoxins, which are secondary metabolites of filamentous fungi and toxic to humans and animals even in small concentrations. These are produced by fungi to reduce the incidence of competitors in environment [8]. The main producers of mycotoxins are fungi of the genus *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium* [9]. Among which we should highlight *Aspergillus* spp. and *Penicillium* spp. because they are the most commonly found in honey. Articles about these microorganisms in honey record these genera in isolated colonies in the United Kingdom, Pakistan, Italy, and Brazil [10–13]. They are also associated with disease in honeybees.

In research performed with honey samples of different blossoming, fungi of different species were isolated, *Alternaria alternata*, *Aspergillus niger*, *Aspergillus proliferans*, *Aspergillus spelunceus*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Daldinia concentrica*, *Emericella discophora*, *Emericella qinqixianii*, *Penicillium corylophilum*, *Penicillium decumbens*, *Penicillium polonicum*, and *Penicillium echinulatum*, of which *P. corylophilum* and *A. niger* were the most frequent, but in low count, indicating that the honey is capable of containing multiplication of these fungi [13]. The presence of fungi does not imply the presence of mycotoxin; it has necessary ideal conditions such as high water activity, the presence of sugars, and the presence of organic acids capable of reducing pH. Necessary conditions for fungal growth are not

always the necessary conditions for production of mycotoxins [9]. As an example, we can cite the patulin produced by species of *Penicillium*, *Aspergillus*, and *Byssochlamys* whose optimum temperature for production is 23–25°C, with minimal water activity of 0.82–0.83. Aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* have ideal temperature of 30–52°C and 0.80–0.95 water activity, and ochratoxin that is produced by species of *Aspergillus* and *Penicillium* needs temperature between 30–35°C and 0.93–0.99 of water activity [9].

Despite of inappropriate condition found in honey for mycotoxin production, it is important to say that the presence of fungus can also cause disease in different ways, as induction of allergic responses and infections. The fungi of genus *Aspergillus* are able to causing bronchopulmonary allergies among other forms of invasive aspergillosis. They are also related in acquired disease by immunocompromised patients in hospital. *Aspergillus fumigatus* is the most pathogenic followed by *A. flavus*, *Aspergillus terreus*, and *A. niger* [14]. The allergies and asthma may be caused by inhaled or ingested spores. For example, *Aspergillus clavatus* and *A. fumigatus* are responsible for allergies from malt workers who inhaled large amounts of spores during the malt handling for contaminated barley [15]. Foods with acidic pH, low humidity, and high concentration of sugars, such as honey, are sources for growth of the fungi *Aspergillus glaucus* [15].

Regarding the *Penicillium*, this fungus was first associated as producer of mycotoxins. They are saprophytic fungi able to grow at water activities less than 0.9; they can invade plants and animals but not as obligate parasite. They have vegetative reproduction by spores. However, the most important aspect concerns the production of toxins as aflatoxins, patulin, and ochratoxins [16]. In humans, only a minority of fungal species has pathogenicity, i.e., *Penicillium marneffei* (Southeast Asia), which is assigned lung infections in people with HIV virus in South Asia and China, and opportunistic infections—keratitis, ear infections, and endocarditis [17].

With respect to yeasts, only *Debaryomyces hansenii*, *Zygosaccharomyces rouxii*, *Zygosaccharomyces mellis*, *Aureobasidium pullulans*, and *Cryptococcus uzbekistanensis* species were isolated from honey [13]. Among them only *Cryptococcus* species was associated with human pathogenicity, i.e., the yeast *Cryptococcus neoformans* is characterized as opportunistic human pathogen able to infect the central nervous system [18].

Among bacteria, *Bacillus* sp. and *Clostridium* sp. were described in honey. *Clostridum perfringens* is known as an enterotoxin producer that happens in final stages of sporulation; thus, in adverse conditions for their development, the toxin will be released together with spore. Vegetative cells also produce enterotoxin but at low levels. Unlike *C. perfringens*, the toxin produced by *Clostridium botulinum* is stronger and produced during propagation. Thus, the best condition for propagation is the same for toxin production, which is 4.5 pH, water activity of 0.93, and temperature varying with strain [19].

There are about 200 species of *Clostridium*; a lot of them has pathogenicity and produce one or more toxins, assimilated by the gut and transported by blood [20]. Only *Clostridium botulinum* was found in honey [2], but was hardly detected with conventional methods; however, with molecular techniques as PCR, the detection was more accurate. In this way, samples that seem

negative showed positive with molecular test [21, 22]. This microorganism enters the beehive through the contaminated water or even by contact of product with ground. This organism does not cause damage to honeybees, but it is responsible for the development of botulism in humans, especially in children or people with weakened immune systems and can lead to death [23].

Genus *Bacillus* comprises rod-shaped Gram-positive bacteria with the ability to form spores. There are 60 species of huge genetic diversity, and most of them are nonpathogenic; the pathogenicity associated with others is in opportunistic form. These pathogens belong to group *Bacillus cereus*, a subgroup *Bacillus subtilis*; however, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus majavensis* can cause poisoning by food too [24]. *Bacillus cereus* is an important pathogen in honey; it is an enterotoxin producer in pH 6.0–8.0 and temperature ranging from 6°C to 21°C, but it is necessary to ingest 10^7 cells/mL to reach toxic effect [19].

Researchers isolated some bacteria in honey samples of different geographical and botanical origins. "They found *B. pumilus* (ML374), *B. licheniformis* (ML103A and ML104B), *B. amyloliquefaciens*, *B. subtilis*, *B. cereus*, *B. thuringiensis*, *B. licheniformis*, *B. megaterium*, and *B. pumilus* [13]." The bacteria of species *B. cereus* are enterotoxin producers; the others of *Bacillus* species are considered safe. Due to their ability of producing bacteriocins, they are promising in the study of new antimicrobial [25].

3. Beneficial microorganisms in honey for humans

Human metabolism is dependent of symbiotic microorganisms, known as the indigenous microflora capable of favoring the production and absorption of essential nutrients to our body such as K and B12 vitamins, pentatonic acid, pyridoxine, and biotin, and acts by modulating the immune system [26]. This microbiota lives in the gut, due to high acidity of the stomach (pH 1.5); the most microorganisms are unable to grow, while in the gut we can found a lot of microorganisms with 500–600 different species [26]. There is no oxygen in gut; for this reason, the gut bacteria are aero-tolerant and facultative anaerobic. We can find bacteria of genus *Actinomyces*, *Bacteroides*, *Clostridium*, *Enterobacter*, *Enterococcus*, *Escherichia*, *Klebsiella*, *Lactobacillus*, *Proteus*, *Pseudomonas*, *Staphylococcus*, and *Streptococcus*; many of them are opportunistic pathogens when move to other parts of the body [26].

For honey production, honeybees ingest nectar and turn this with help of enzymes. Beyond the enzymes, they incorporate some symbiont microorganisms associated with gastrointestinal tract that can bring benefit to human health [27]. The natural human microbiota is stable; so it is necessary for daily intake of the new symbiont to be able to populate the human body and maintain its benefits [28]. These microorganisms are known as probiotics and, when they grow in human gut, can make nourishment benefits, like fermentation, and broke nutrients facilitating absorption of short-chain fatty acids, ions, amino acids, and vitamins; protective effect, preventing invasion of pathogenic microorganisms; and trophic effect in the gut epithelium and in the system [28].

Bacterium *Gluconobacter oxydans* was isolated from honey harvested directly from beehive. Also, *Pseudomonas* spp. and *Bacillus* spp. were found [29, 30]. However, *G. oxydans* is highlighted because they showed 100% of survival in pH of 5.0 and 50% of survival after 3 h of contact in pH of 2.0 and showed resistance in 2% of bile salts. This is atypical behavior for bacteria, because normally they have low resistance in acidic environments. For this way, a bacterium resistant to condition of the stomach is promising to arrive in the gut, where it will grow and will make benefit [29]. These bacteria can assimilate cholesterol reducing absorption of this component by the body, and it can be used as probiotics in food [29]. In addition to this, as honey is rich in fructose, some bacteria that live in there possess the ability to degrade fructose more easily; these bacteria are known as fructophilic lactic acid that prefer to metabolize fructose and not glucose as normally is observed. In the gut, these bacteria produce bacteriocins that act as a barrier to other microorganisms and contribute to the immune system. *Lactobacillus kunkeei*, fructophilic lactic acid bacteria, were found in the stomach of honeybees, as well as in their hives [27].

Besides these microorganisms is necessary consumption of substances that promote their development, known as prebiotics. These prebiotics are components, like oligosaccharides, that are not digested by humans, but they serve as a substrate for the growth and performance of probiotics [28, 31]. Currently, there is a great interest in combining probiotics with oligosaccharides acting like prebiotic. There are studies with probiotic *Lactobacillus* sp., which show that when they are grown in the presence of oligonucleotides, they show an increase in growth and antibacterial activity with production of bacteriocins [32].

The most-studied prebiotics are fructo-oligosaccharides, inulin, and oligofructose especially [33, 34]. However, there are others recognized as prebiotic, like galacto-oligosaccharides, trans-galactosylated oligosaccharide, isomalto-oligosaccharides, lactulose, pyrodextrin, and soy-oligosaccharides [28]. In honey we can find malto-oligosaccharides [35], specifically in Brazilian honey samples that were found in isomaltose, cellobiose, panose, maltotriose, melezitose, raffinose, maltose, turanose, and maltotriose, which are characterized as prebiotics [36].

In addition to probiotics, there are microorganisms associated with honey that can produce bacteriocins, which are substances able to reduce or eliminate competing microorganisms. These are peptides produced by bacteria producers of lactic acid, to reduce competition for nutrients, making inappropriate environment for development of other bacteria; for this reason, they are studied as an option for replacing antibiotics, and as usual these can cause harsh effects to humans also. Bacteriocins have high potency *in vivo* and *in vitro* and have low toxicity, and they can be produced in situ through consumption of probiotics or purified through bioengineering [37]. In 2013, a study was conducted with a new bacterium strain isolated from honey, able to produce bacteriocins fungicides called *Bacillus* BH072. These bacteriocins were tested and showed inhibitory character against *A. niger* CGMCC3.03928, *Fusarium oxysporum* CGMCC3.2830, *Pythium*, and *Botrytis cinerea* CGMCC3.4584 [25]. In another search, 13 lactic acid bacteria were isolated from honey and honeybees, and they were tested against bovine mastitis; they observed that the synergism between lactic acid bacteria and honey was able to inhibit growth of bacteria that cause mastitis, even those that were resistant to other antibiotics, and this is a promising preventive treatment to be studied [38].

Studies suggested that the antimicrobial character of honey is attributed to activity of these bacteria in honey; these are also present in the stomach of honeybees. *Lactobacillus* spp. were isolated from the stomach of honeybees and honey, they were then tested against *Escherichia coli* and *Salmonella enterica*, and they showed inhibitory effect. It is important to say that *Lactobacillus helsingborgensis* and *L. kunkeei* are the most candidate promisors like probiotic producers of bacteriocins [39]. Direct application of honey was also effective against *Serratia marcescens* and *Candida albicans* [40]. Beyond health benefits, discovery and application of microorganisms able to develop biotechnological products must be studied because they can improve lifestyle and human survival, becoming in this way beneficial microorganisms.

Besides the microbiota associated with honey, it is worth mentioning that this product alone is highly beneficial by features from its composition. This makes the honey effective activity like antimicrobial, antioxidant, anti-inflammatory, anticancer, antihyperlipidemic, cardioprotective properties, for ocular treatment, gastrointestinal tract disorders, neurological disorders and wound healing [1]. Honey has a series of phenolic acids like caffeic, ellagic, ferulic, and p-coumaric acids; flavonoids, such as apigenin, chrysin, galangin, hesperetin, kaempferol, pinocembrin, and quercetin; and antioxidants, such as tocopherols, ascorbic acid, superoxide dismutase, catalase, and reduced glutathione [41]. These compounds are known for their ability to reduce free radicals; this composition may vary depending on floral source that honeybees have visited for honey production [42]. Its antimicrobial activity makes it an important substance for the treatment of wounds as a result of carbon, lipids, amino acids, proteins, vitamins, and minerals active in healing. Components such as hydrogen peroxide, high osmolarity, acidity, non-peroxide factors, nitric oxide, and phenols are active in their healing effect. It also promotes growth of tissue in the human body, and it has anti-inflammatory activity [43]. However, it is important to note that honey directed to the treatment of wounds and inflammation should undergo irradiation treatment, so that microbiota will not interfere negatively on treatment [44].

Finally, it is important to note that consumption of foods able to bring health benefits, beyond nutrition, is a current practice that should be encouraged; honey is characterized as such, and it should be ingested daily.

4. Microorganisms in honey for industrial use

The yeasts that were found in honey are able to withstand high concentrations of acids and sugar, and it can be a problem for the honey processing industry; however, they are promising for fermentative processes. Furthermore, the low concentrations of these nutrients in honey characterize yeasts as nutritionally less demanding. *Saccharomyces* is widely found in honey, as well as *Rhodotorula*, *Debaryomyces*, *Hansenula*, *Lipomyces*, *Oosporidium*, *Pichiu*, *Torulopsis*, *Trichosporon*, *Nematospora*, *Schizosaccharomyces*, *Schwanniomyces*, *Torulu*, and *Zygosaccharomyces*. The amount of these yeasts will be increased in relation to the humidity of honey; honey with higher humidity, we will have higher population of yeasts [2]. Species of *Zygosaccharomyces* are recognized as osmophilic; *Zygosaccharomyces gambellarensis* (a new species of yeast),

Zygosaccharomyces favi sp. nov., and *Zygosaccharomyces clade* were isolated from honey and bee-bread. They are obligatory osmophilic, and they do not have the ability to grow in high water activity [45]. In another study, during the isolation of 20 strains of yeasts from honey, all of them were characterized as *Zygosaccharomyces rouxii* [46]. Studies show that this yeast has high productivity of glycerol, a common characteristic in osmophilic yeast [47].

Besides yeast, filamentous fungi are also significant because they are known for their ability to produce extracellular substances such as enzymes and acids; they must be studied, as they are able to produce substances of industrial interest in osmotic stress condition. The genera *Aspergillus* and *Penicillium*, previously mentioned pathogens [10], are able to produce numerous extracellular compounds with biotechnological importance due to their characteristic of digest food externally before absorption of nutrients; for this reason, they produce organic acids and extracellular enzymes such as amylases and citric acid [15]. These fungi are capable of degrading starch, hemicellulose, cellulose, pectin, and sugars among other polymers. Some of them are able to degrade fats, oils, chitin, and keratin [16, 48].

5. The gut microbiota as an environmental factor for honeybee health

Honeybees have a beneficial anaerobic and micro-aerobic natural microbiota acquired and installed in their body. This includes Gram-negative groups like species *Gilliamella apicola*, *Snodgrassella alvi*, and *Frischella perrara* and Gram-positive groups like species of *Lactobacillus* and *Bifidobacterium* [49, 50]. That is, *Acetobacteraceae*, *Parasaccharibacter apium* confers resistant to *Nosema* [51] and *Bartonella apis*, a honey bee gut symbiont of the class *Alphaproteobacteria* [52, 53]. So it is natural for bees to acquire these microorganisms through feeding [49]. This honeybee normal microbiota comes from food, pollen, and honey consumption or through contact with other worker honeybees.

The microbiota associated to the honeybee *A. mellifera* is complex, and it has been described as being mainly composed of yeasts, Gram-positive bacteria (such as *Lactobacillus rigidus apis*, *S. constellatus*, *Bacillus* spp., *Streptococcus*, and *Clostridium*), and Gram-negative or Gram-variable bacteria (*Achromobacter*, *Citrobacter*, *Enterobacter*, *Erwinia*, *Escherichia coli*, *Flavobacterium*, *Klebsiella*, *Proteus*, and *Pseudomonas*) [54–58].

There are several bacterial species negatively affecting honeybee health—*Paenibacillus larvae*, *Melissococcus plutonius*, *Spiroplasma apis*, and *Spiroplasma melliferum* [59–61]. Besides bacteria, there are many fungi, viruses [62], and protozoa, i.e., *Apicystis bombi*, *Crithidia mellificae*, and *Lotmaria passim* (**Figure 1**) [63–65]. *P. larvae* is a sporulated Gram-positive *Bacillus* that causes the American foulbrood disease in larvae.

Gilliam reported that these bacteria could be endemic of the digestive tract of adult honeybees and independent of seasons and nutritional factors [11]. They are different depending on the sources of nectar and the presence of other bacterial genera in the stomach of the honeybee. It seems that bees and lactic acid bacteria developed mutualism. Lactic acid bacteria prepare the environment to make nutrients available for honeybees; on the other hand, intestinal tract

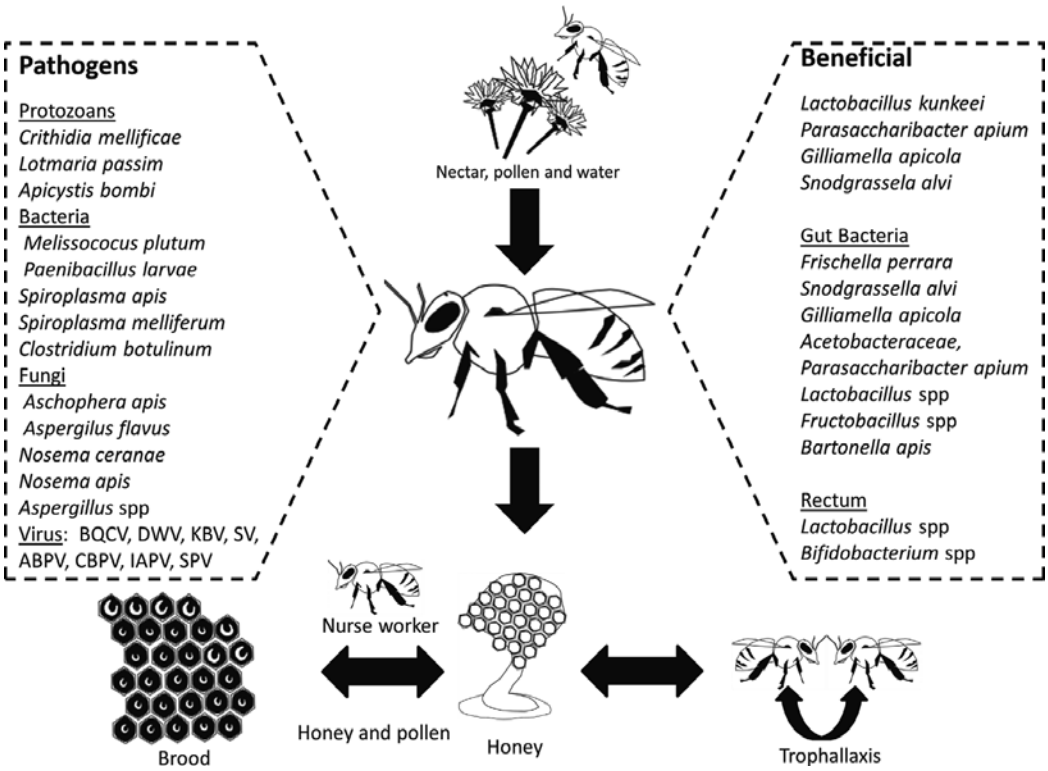

Figure 1. The pathogens and beneficial microorganisms in honeybee: one pathway of bee food contamination comes from environmental nectar, pollen (on flowers), and water collected by worker honeybees. The food is stored in beehive and can be transferred by trophallaxis among workers and brood. Another pathway is the consumption by honeybees of contaminated honey and/or pollen from other beehives. Common viruses: black queen cell virus (BQCV), deformed wing virus (DWV), Kashmir bee virus (KBV), Sacbrood virus (SV), acute bee paralysis virus (ABPV), chronic bee paralysis virus (CBPV), Israel acute paralysis virus (IAPV), and slow paralysis virus (SPV). Niche of beneficial microbiota on alimentary tract (gut). The arrows indicate the transfer of microorganisms by food among individuals (larva and adults) in the beehive. For detail, see in the text.

of honeybees is protected from harmful microorganisms. The honeybee regurgitates the nectar stocked in the crop in the hive honeycomb that has an optimum temperature of 35°C [66] for the development of lactic acid bacteria.

The honeybee larvae probably are sterile initially, but as feed on honey from nurse workers, honeybees gain over time this intestinal flora before completing their life cycle [67]. Honeybees harbor a number of commensal or beneficial bacteria distributed throughout the different compartments of their gastrointestinal tract. Each compartment of the honeybee gastrointestinal tract has a distinct environment favoring specific microorganisms [68]. Several findings have indicated that the honeybee gut is colonized by a distinctive set of bacterial species designated as the core gut microbiota [69]. Because the community composition changes through the life cycle of honeybee, the colonization of the gut is believed to be influenced by the age [68]. During the course of their life span, worker honeybee performs many different tasks that can contribute to these variations. Newly emerged worker honeybees nurse larvae within the hive, whereas

older worker honeybees build and maintain the wax combs, defend the colony, and receive and process food that is collected by foragers. In addition to the microbiota in the gut, a novel lactic acid bacterial flora composed of 13 taxonomically well-defined *Lactobacillus* and *Bifidobacterium* species were discovered in the crop of honeybees [70, 71]. The crop functions as an inflatable bag that can transport the nectar back to the hive for storage and honey production. It is hypothesized that lactic acid bacteria play a key role in the conversion of both nectar to honey and pollen to beebread (stored food rich in protein) due to their fermentation properties [70, 72]. The lactic acid bacterial microbiota is of great importance to the honeybee health, protecting them against bee pathogens [73, 74] and contributing to the antimicrobial properties of honey [71].

Lactic acid bacteria are found in two distinct phyla: *Firmicutes* and *Actinobacteria*. The most important genera of lactic acid bacteria within the *Firmicutes* are *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella*, which all have a low G+C content. Lactic acid bacteria in the *Actinobacteria* phylum only include species of the *Bifidobacterium* genus that in contrast to the *Firmicutes* members have a high G+C content [75, 76].

Lactic acid bacteria are important inhabitants of the intestinal tract of man and other mammalian and vertebrate animals. *Lactobacillus* and *Enterococcus* are members of this family and are also present in food and fermentation processes [77]. These microorganisms disclose interesting properties not only for the food industry but also for health. The antimicrobial potential of these bacteria includes, among others, the synthesis of compounds such as lactic acid, short-chain volatile fatty acids, and bacteriocin-like molecules [78, 79]. Antagonistic studies are generally directed toward food spoilage and/or pathogenic microorganisms related to the host or product from which the lactic acid bacteria were isolated. Fructophilic lactic acid bacteria are a special group of lactic acid bacteria, which prefer fructose over glucose as growth substrate [80]. They are found in fructose-rich niches, e.g., flowers and fruits. Moreover, the microorganisms can be found in fermented foods made from specific fruits, including wine, fermented cocoa beans, and fermented durian-based condiments [81–83]. *Fructobacillus* spp. and *L. kunkeei* are representatives of these microorganisms, and a few novel species have recently been classified as members of this interesting group [84, 85].

Quite recently fructophilic lactic acid bacteria were found in the gastrointestinal tract of several flower- or fructose-related insects, including honeybees, tropical fruit flies, and giant ants [86–88], whose diets are fructose rich. Of these insects, honeybees are economically and agriculturally important for honey production and especially for crop pollination, which links to human food production. However, despite the importance of these insects in nature and in our lives, populations of honeybees are reported to have decreased considerably during the last decade and to be still decreasing worldwide, mainly by colony collapse disorder [89]. To understand and to prevent the disorder, microbial interactions, both symbiotic and pathogenic, have recently been studied [90, 91], and findings have indicated that honeybees carry specific microbiota dissimilar to other animals, including humans. Fructophilic lactic acid bacteria, especially *L. kunkeei*, have been found to be one of the dominating bacterial species in several honeybees kept or captured in different regions [73, 90]. Lactic acid bacteria have been successfully applied as probiotics to contribute to health in humans and various companion and farm animals [92, 93]. As lactic acid bacteria are important components in their gastrointestinal tract,

with a reported impact on the intestinal barrier mechanism [94], it is not surprising that lactic acid bacteria, especially fructophilic lactic acid bacteria, may be involved with honeybee health.

Symbiosis is common in nature, in which symbionts as commensals or mutualists evolved to benefit each other. Culture-independent studies of the human microbiota identified recently a complex symbiotic environment with more than 1000 bacterial phylotypes representing more than 7000 strains [95]. The composition of this microbiota has been suggested to be a result of a highly coevolved symbiosis and commensalism influenced by nutrition, physiology, and immunological factors. It varied with the sources of nectar and the presence of other bacterial genera within the honeybee and ended up eventually in the honey (**Figure 1**).

6. Microorganisms in stingless bee honey

Products of stingless bees are consumed since before the discovery of the Americas to the present day. Honey of these bees has activities against microorganisms, having importance in the colony maintenance as a microbiologically stable environment [96]. Stingless bee honey has characteristics that confer antimicrobial character, i.e., activity against Gram-negative and Gram-positive bacteria such as *Enterococcus*, *Staphylococcus faecalis*, *Staphylococcus aureus*, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Candida albicans* [1, 97, 98], which justifies its use in popular medicine [6, 41, 99–101].

However, *Meliponini* also feature mutualistic interaction with microorganisms, i.e., lactic acid bacteria are found in Australian species as *Tetragonula carbonaria*, *T. hockingsi*, and *Austroplebeia australis* [102]. Yeasts such as *Starmerella meliponinorum*, *Starmerella neotropicalis*, *Candida apicola*, and *Zygosaccharomyces* spp. are commonly found in the Neotropical species of stingless bees such as *Tetragonisca angustula*, *Frieseomelitta varia*, *Melipona quinquefasciata*, and *Melipona quadrifasciata* [103–105] and provide sensory and conservation to food characteristics [106–109].

About fungi, the interesting fact is that bees cultivate them as food [110] and protection against other pathogenic microorganisms [111], i.e., *Scaptotrigona aff. depilis* young larvae, needs to be fed from the mycelium of *Monascus* genus (*Ascomycotina*) to complete their development [112], which reinforces the intrinsic evolutionary relationship between microorganisms and these bees.

Little is known about pathogens in stingless bees; however, there are no pathogen transfer record from *A. mellifera* [113], which shows the lack of information about microorganisms in *Meliponini*.

7. Microorganism detection methodologies in honey and honeybee products

7.1. Microbial diversity

Much has been discussed about the succession of gut microbiota among queens, workers, and larvae and the role of the diversity on the quality of honey, safety, and health of the colony [11, 53, 114–117]. New methodologies have made it possible to access information about the

differences in the profile of this microbiota in different apiculture sources [118–121], species [53, 122] and genetic diversity [116] of honeybees, development stages [53, 68, 117, 122–126], nutrition [116, 127], location inside the gut [49, 53, 68] and digestive system [120], ontogenetic stage and geographic location [118, 122, 125], environmental conditions [128], health control [129], and individual [116, 125].

This access has been carried out mainly by sequencing the coding region of the 16S subunit of the bacterial ribosome [53, 121, 130], both from genomic DNA from microorganisms growing on selective media as Man-Rogosa-Sharpe agar, Sabouraud dextrose agar, and *Candida* agar [117, 120, 131, 132], such as process-independent culture as specific PCR [68], denaturing gradient gel electrophoresis [124, 125], mixed and deep 16S sequencing [49, 128], pyrosequencing [53, 116, 121], and clone library [115, 118, 120, 122]. While culture-dependent methods are ideal for quantification of microorganisms and phenotypic testing, culture-independent methods generally have greater coverage in relation to the amount of different species accessed and are ideal for fingerprinting studies, and the identification of these species may be performed by real-time PCR analysis [49, 68, 125, 128]. These methods, although they have different principles, were able to distinguish similarly the narrow niche of bacterial species and the diversity of strains present in these matrices [120]. In some works, the complete genome [132, 133] or metagenome [114, 115] of the narrow range of species of microorganisms is accessed, enabling the search for specific functions of these bacteria for beehives by gene annotation, PCR screening [114], and Post-Light TM ion semiconductor sequencing [127]. Fluorescent in situ hybridization microscopy has also been used to characterize distribution and abundance of specific phyla across the life cycle and among gut organs [68]. Changes in the diversity of microbial populations found by these authors would be able to explain the transformations that occur in honey and pollen, as well as strategies of these insects to combat pathogens and invaders [11, 114, 116, 121] and beebread preservation [11, 120].

Several microorganisms present in the honey and in the gut of honeybees have antagonistic effects on honeybees and human pathogens, especially of *Bacillus* genus [123, 134], lactic acid bacteria as *Lactobacillus* [71, 121, 124, 130–132, 135], *Enterococcus* [130], *Bifidobacteria* [116, 132, 136–138], and *Acetobacteraceae* [117, 121, 133]. These same microorganisms can be accessed for other purposes, such as its potential as fermenters [116, 130, 133] or probiotics [116]. In this case, direct detection strategies of these microorganisms are not the analysis priority since their isolation is of interest to researchers for the antagonism studies. "This isolation is mainly done using traditional selective media, especially Man-Rogosa and Sharpe agar to *Lactobacillus*; *Streptococcus* selective medium and MTPY or Wilkins-Chalgren medium for *Bifidobacterium*" [71, 130, 136, 138, 139], with or without prior enrichment [133], and the identification of the isolates is mainly performed by sequencing 16S rRNA amplicons. However, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry profiling was used for acetobacterium identification from bumble bee crop [133] and clustering of lactic acid bacteria of a bumble bee gut microbiota [139]. Several studies have shown the effectiveness of these microorganisms to inhibit human pathogens such as *Staphylococcus aureus*, *Escherichia coli* O157: H7, *Salmonella*, and *Listeria* [130, 140] or pathogens of honeybees as *Melissococcus plutonius* [124, 138], the causative agent of European foulbrood and *Paenibacillus*

larvae [123, 124, 130, 134], and the causative agent of American foulbrood, among others. This effectiveness is generally associated with the production of acid, bacteriocins [130], and other antimicrobial molecules [140].

7.2. Monitoring of the microbiological honey quality

Traditional methods are often still used for monitoring the microbiological quality of honey used for human consumption, even as the rates established by the laws use these methods. Potato dextrose agar and yeast extract glucose chloramphenicol agar are media normally used for aerobic count and the total fungi (yeasts and molds), while Violet Red Bile and MacConkey medium agars are normally used for counting coliforms, which can also be done by the most probable number technique [119, 141, 142]. These media have recently been used to monitor the efficiency of a new filter-based method based in reducing the microbial burden and to improve the microbiological quality of honey [143]. Potato glucose agar in Brazil was also used for monitoring the honey contamination by yeast and fungi [144]. Standard plate count agar is used for monitoring of mesophilic bacteria, such as that was done in honey samples of Portugal [141, 142] and Argentina [119, 145].

7.3. Detection of honeybee pathogens in honey

The honey is an important route of contamination of honeybees, spreading many microorganisms, particularly pathogens that infect the honeybees. Several molecular techniques have been developed for the detection of pathogens like *Paenibacillus larvae*, *Melissococcus plutonius*, *Nosema ceranae* and *Nosema apis* [129, 146, 147], *Ascophera apis* and *Ascophera ceranae*, and *A. flavus* [129, 148]. Among them can highlight the simple PCR [149–151], NESTED-PCR [152], RT-PCR [153, 154], immunology-based tests (ELISA), and probe-based hybridization analysis [155]. The main advantages of these techniques would be less needed for sample treatment which often can be applied directly to the honeybee products, fast technique, specificity, and sensitivity of detection.

The use of these techniques and the detection of this pathogen have allowed the control of mortality of honeybee populations around the world, restricting the dissemination of pathogens in bee products. For example, the diagnosis of American foulbrood and European foulbrood usually occurs through visual inspection of brood combs and detection of diseased larvae, subjective criteria that could be confused with other beehive conditions [155, 156]. The traditional methods of detection of these pathogens include the visualization by microscopy and detection in tissues [155]; culture on selective medium [151, 155, 156], including *P. larvae* agar [151]; bacteriophage sensitivity; immunotechniques; and microscopy of suspect bacterial strains have been considered adequate for routine identification purposes [151]; these methods are time-consuming and laborious but especially require that the infection is in progress so that the pathogen is detected and confirmed. The detection of pathogens before any clinical signs of disease to be visible in the colony would not only control these diseases but also the prevention of their consequences for the hive. That is, *M. plutonius* was detected in healthy colonies by RT-PCR in England and Wales, showing that the extent of the prevalence of this pathogen in hives goes beyond the clinical signs [157].

RT-PCR has been used to simultaneously detect multiple viruses such as in cases of honeybee parasitic mite syndrome where five out of seven viruses were detected in sample mite in Thailand [158]. Also, different multiplex RT-PCR were developed for the simultaneous detection of i) black queen cell virus (BQCV), deformed wing virus (DWV), Kashmir bee virus (KBV) and Sacbrood virus (SV) [159], ii) acute bee paralysis virus (ABPV), BQCV and SV [160], iii) ABPV and SV [161] iv) ABPV, chronic bee paralysis virus (CBPV), BQCV, DWV, KBV, and SV [162]. The effectiveness of this method in the detection of these pathogens was demonstrated in the simultaneous detection of these viruses in colonies [159, 160] and queens [162], where up to 93% of the queens have multiple infections [162].

Even more efficiently nine viruses (ABPV, BQCV, CBPV, DWV, KBV, SV, Israel acute paralysis virus (IAPV), *Varroa destructor* virus 1 (VDV-1), and slow paralysis virus (SPV)) were detected simultaneously in a single test developed by Glover and coworkers. These authors used a microarray technique with oligonucleotides based on DNA sequences of each of these viruses, but the time and cost of the technique are still unfeasible with its use for routine diagnosis [163].

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Chapter 12

Techniques for the Evaluation of Microbiological Quality in Honey

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Additional information is available at the end of the chapter

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Abstract

The aim of this chapter is to describe the most commonly used techniques to evaluate the microbiological characteristics of honey for the purpose of identifying its contaminant flora, its significance and its control in this type of food. Honey is a product that is rich in simple sugars, minerals, vitamins and bioactive compounds and possesses an antimicrobial activity of great significance for human health. However, as it has physical and chemical properties that are unfavourable for the proliferation of micro-flora, honey can contain a large population of microorganisms from two sources of contamination—the first primarily represented by pollen, the digestive system of the bee, dust, air and the flower itself; and the second as the result of negligence and the absence of good health practices during handling and use; for example, placing honey in wooden beehives directly on the floor or the use of improperly washed honey extraction equipment, rather than equipment based on the oxidizable material, or using very dark honeycombs and storing the honey for long periods in wooden beehives. As honey is a natural product, the risks inherent to the lack of industrial processing, such as pasteurization and strict microbiological quality control, are often overlooked.

Keywords: microbiological analysis, microbiological standards, good habits, antimicrobial action, antimicrobial agents

1. Introduction

Most of the analysis techniques described in this chapter were recommended by the Agência Nacional de Vigilância Sanitária (the National Health Surveillance Agency) (ANVISA) [1], including the Official Analytical Methods for the Microbiological Analysis and Control of Products from Animal and Water Sources [2], those of the American Public Health Association; described in the fourth edition of the Compendium of Methods for the Microbiological Examination of Foods [3–5], the International Commission on Microbiological Specifications for Foods [6, 7]; the Food and Drug Administration (FDA), recommended by the Ministry of Agriculture for the analysis of foods of animal origin, in accordance with Normative Instruction number 62, dated August 26th, 2003 [2]; the Food Safety and Inspection Service of the US Department of Agriculture [8], the Association of the Official Methods of Analysis of AOAC International [9], the Bacteriological Analytical Manual (FDA) [10], the Microbiology Laboratory Guidebook [11] and the latest editions of the International Organization for Standardization [12, 13].

Among the various parameters that indicate the quality and safety of honey, the most important are those that define its microbiological characteristics. Honey, as with any other raw material of vegetable or animal origin, naturally presents microbial contaminants of commercial importance formed by microorganisms adapted to the characteristics of the honey, such as high-sugar content, low acidity and the presence of natural antimicrobial substances. Because of these characteristics, the microbial load in honey is generally low, below 10^2 CFU/g, and can even reach 10^3–10^4 CFU/g. Consequently, it can cause undesirable changes by reducing the shelf-life of the product. It presents floral indicators of the possible presence of pathogenic microorganisms, and so can be harmful to the health of the consumer. Protecting food products from any kind of contamination or adulteration which can cause harm to public health or economic disorder is a global concern [8] and specific methods of analysis are required to evaluate this type of raw material. Moreover, the risks represented by the poor handling conditions used by workers responsible for the harvest, extraction and preparation of this product require effective interventions and procedures to minimize these risks [14].

Aiming to control the quality of honey, the World Trade Organization recommends the adoption of standards, guidelines and norms developed by Codex Alimentarius—the revised codex standard for honey 2001 [15]. This is an international public agency created by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) [16], both of which form part of the United Nations Organization (UNO). MERCOSUL GMC resolution n° 15 1994 approved the Technical Regulations for the Identity and Quality of Honey, based on resolutions n° 18 (1992) and n° 91 (1993) of the Common Market Group [17], in which honey can contain a maximum of 100 colony forming units of fungus per gram (CFU/g). Normative instruction nº 11 approved, on 20 October 2000, the Technical Regulations for the Identity and Quality of Honey [18] and normative instruction nº 3 dated January 19th 2001 approved the Technical Regulations for the Identity and Quality of bee apitoxin, beeswax, royal jelly, lyophilized royal jelly, bee pollen, propolis and propolis extract [19], as previously microbiological standards had not been established for these apiculture products. To ensure the

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credibility of the results however, some steps must be observed. The methods in this chapter are described as simply as possible in order to be accessible to fully qualified professionals, lab technicians and students with varying levels of education and training. This chapter provides comprehensive material presented in a didactic manner, with texts and diagrams that facilitate understanding. The basic techniques of microbiology described are accompanied by a brief overview of the microorganism researched in order to provide a solid theoretical basis, which will be of great value for understanding the method and interpretation of results. This chapter, therefore, aims to present the most commonly used techniques for assessing the microbiological characteristics of honey to identify its contaminant flora, its significance and its control in this type of food.

1.1. Sampling plan for the microbiological analysis of honey lots

Sampling plans allow the evaluation of the microbiological conditions of honey lots. These were proposed by the International Commission on Microbiological Specifications for Foods [6] and their application supports the acceptance or rejection of a honey lot as a whole, describing the hygienic sanitary conditions under which this food was obtained, processed, stored, distributed for consumption, as well as its shelf life and the risk posed to consumer health.

For the microbiological analysis of a honey lot, it is necessary to define some important concepts, such as: lot, *n, c, m* and *M*. A 'lot' is the total units of honey pots produced, handled or stored under the same conditions, within a certain period; 'n' is the number of units taken randomly from a lot to be analysed individually. For honey, '*n*' is equal to five sample units and constitutes a representative sample of the lot; '*m*' is the set of microbiological standards established for a microorganism in a given food; '*c*' is the maximum acceptable number of units in which microbial counts in the lot are above the minimum threshold (*m*) and below the maximum tolerated limit (*M*); ' *M*' is the tolerable limit, above standard, which can be reached by (*c*) sample units, but cannot be exceeded by either [6].

Brazilian legislation on the microbiological requirements of food includes Ordinance nº 101 of 1993 of the Ministry of Agriculture, Livestock and Supply and RDC-12 Resolution 2001 of the National Health Surveillance Agency of the Ministry of Health [1]. In the case of honey (molasses and similar) a value of $n = 5$ is adopted, while values of *c, m* and vary according to the microorganism considered: coliforms at 45°C/g ($n = 5$, $c = 2$, $m = 10$ and $M = 10^2$) and *Salmonella sp*/25 g (n=5 $c = 0$; m = absent) under this legislation are more flexible than the levels established by Mercosul [17] in which honey must meet the following microbiological characteristics: Coliforms at 35°C/g ($n = 5$, $c = 0$, $m = 0$); *Salmonella* spp - *Shigella* spp/25 g ($n = 10$, $c = 0$, $m = 0$); Fungi and Yeast CFU/g ($n = 5$, $c = 2$ m=10, $M = 100$). Therefore, a maximum of 100 colony forming units of fungus per gram of honey (CFU/g) is acceptable.

For the analysis of *Salmonella sp*/25 g, a two-class plan is applied, as this trial investigates the presence or absence of this microorganism. In this case, '*c*' is equal to zero, absence is acceptable and the presence of any sample unit is unacceptable. In these tests a single sample analysis is performed. For analysis of coliform 45°C, a three-class plan is applied, which classifies lots into three categories: acceptable, intermediate and unacceptable. In this case, the standard is

not absence but values within a range between *m* and *M*. In the two-class plan, *M* separates acceptable from unacceptable lots. In a three-class plan this value separates an acceptable lot from an intermediate lot [10, 11].

1.2. Transport of samples

Samples of food concentrates such as honey are microbiologically stable and can be transported and stored at ambient temperature. Nevertheless, they should be protected against moisture and excessive heat [20].

1.3. Analytical unit

This is the amount of food sample used in conducting one or more tests. The sample unit must be greater than that required for analysis, with sufficient quantities for the counter-sample.

In Brazil, tests for the quantitation of microorganisms in honey comprise mould and yeast counts, the count of total and faecal coliforms and S*almonella* analysis, the trials of which are usually done with an analytical unit of 25 g of honey (in special cases at least 10 g of honey can be used). Analytical units of 25 g meet the requirements of ISO 6887-1 [20], and those of the Compendium, for all tests. Two analytical units are required for analysis of a honey sample—one for mould and yeast quantification, total and thermotolerant coliform count and the other test for the absence or presence of *Salmonella*.

1.4. Homogenization of the honey sample and withdrawal of the analytical unit

Disinfect the area outside the packaging with 70% ethanol and remove the jar lid aseptically. Observe and note the presence of abnormalities in the packaging or in the internal content such as bloating, leakage, the presence of foreign bodies, odour and/or strange appearance.

Before the withdrawal of analytical units, the content of the sample should be homogenized to ensure that the removed portion is representative of all the material. In the case of honey in a jar with enough room for agitation, the package should be inverted 25 times. If there is no free space for agitation, use a second sterile vial and transfer the sample from one vial to another three times. Remove the analytical unit with a sterile spatula (ISO 6887-5: 2010) [21].

2. Description of methods

To increase the reliability of the results obtained, all tests should be performed in triplicate, following the methods described below.

2.1. Total and thermotolerant coliforms

The bacteria are in Gram-negative bacilli form, are facultative, not sporogenic anaerobes, capable of fermenting lactose with gas production, and are temperature dependent. Total coliforms, also known as coliforms at 35°C, are a sub-group of the *Enterobacteriaceae* family.

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The second edition of Bergey's Manual of Systematic Bacteriology [22] includes 44 genera and 176 species in this sub-group. The total coliform group includes only enterobacteria that can ferment lactose with the production of gas, for 24–48 hours at 35°C. More than 20 species fall into this category, including bacteria originating from the gastrointestinal tract of humans and other warm blooded animals such as *Escherichia coli*, and non-enteric bacteria such as *Citrobacter*, *Enterobacter*, *Klebsiella* and *Serratia*, among others [23].

Lactose fermentation capacity is analysed through the formation of gas and/or acid in the lactose-containing culture media. These characteristics are used in traditional methods of total coliform counting.

With modern methods, it is possible to directly detect the activity of the β-galactosidase enzyme involved in the fermentative metabolism of lactose, incorporating the substrates for the enzyme in culture media. One of these substrates is ONPG (ortho-nitrophenyl-β-D-galactopyranoside) which when degraded by β-galactosidase results in a product that is yellow in colour. It also possesses the X-GAL (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) substrate, which results in a product with an intense blue staining, and Salmon-Gal (6-chloro-3-indolyl-β- D-galactopyranoside), whose degradation product is a salmon red colour [23, 24].

The thermotolerant coliform group, also known as coliforms at 45°C but usually called faecal coliforms, is a subgroup of total coliforms which are restricted to members capable of fermenting lactose in 24 hours at 44.5–45.5°C with gas production [23–25]. While this definition aims in principle to select only enterobacteria that originate from the gastrointestinal tract (*E. coli*), it is currently known that the group includes members of non-faecal origin (various strains of *Klebsiella pneumoniae, Pantoea agglomerans, Enterobacter aerogenes, Enterobacter cloacae* and *Citrobacter freundii*) [23].

Escherichia coli have as their natural habitat the intestinal tract of warm-blooded animals, but can be introduced into food from non-faecal sources. They can grow in eosin methylene blue agar where their growth characteristics allow them to be distinguished from other coliforms [23, 25–27].

These bacteria by themselves do not generally represent a major risk, but can indicate poor quality food that may contain harmful agents. According to the International Commission on Microbiological Specifications for Foods [7], total coliforms, thermotolerant coliforms and *Escherichia coli* are microorganisms with a low or indirect risk to health. Their presence may indicate inadequate hygiene and sanitation, demonstrating failures during post-processing, as they are easily inactivated by sanitizers and heat treatment.

Method: This is based on the most probable number (MPN) technique involving inoculation in tubes with lauryl sulphate tryptose broth (LST). This technique is the most used for coliform-bacteria counting. The most probable number in a sample is determined by using a confidence interval table at 95% probability for the various positive tube combinations in three or five tube series [27, 28].

This method enables the density of the viable organisms present in a sample under analysis to be estimated and is based on the principle that the bacteria present in a sample can be separated by agitation, resulting in a suspension of bacterial cells, evenly distributed in the sample.

It is based on the inoculation of an increasing sample volume in a suitable culture medium for the growth of microorganisms, with each volume being inoculated into a series of tubes. Inoculum is obtained by sampling successive dilutions, the streaking of which provides positive and/or negative results allowing the calculation of the density of bacteria investigated by the application of probability calculations.

According to the methods studied, total and thermotolerant coliform and *E. coli* counting by the most probable number method is conducted in four steps [5, 7, 9, 23, 26–28]:

(1) Presumptive test for total coliforms: using lauryl sulphate tryptose broth the observation of growth with gas production is considered suspect (presumptive) for the presence of coliforms. The presence of the surfactant in the lauryl sulphate tryptose broth inhibits the growth of the cytoplasmic membrane of Gram-positive bacteria and enables the presence of lactose fermentation, which releases carbon dioxide. The presence of this gas is evident in the Durham tube.

(2) Confirmation of total coliform test: using brilliant green bile broth (BGBB) there is notable development of bacteria of the coliform group, which is again confirmed by the formation of gas. This occurs because this broth is selective due to the presence of bovine bile and a triphenylmethane dye derivative which inhibits Gram-positive bacteria and sporulated lactose fermenting bacteria. This step of the examination reduces the possibility of false positive results arising from the activity of sporulated bacteria and Gram-positive lactose fermenting bacteria. Observation of growth through gas production in brilliant green bile tubes is considered confirmatory for the presence of total coliforms.

(3) Confirmation test for thermotolerant coliforms: this method uses *Escherichia coli* broth (EC) containing lactose, a selective medium containing a mixture of phosphate which maintains the pH of the medium at an appropriate amount. This selectivity is due to bile salts, which inhibit the growth of the Gram-positive microorganism. If there is gas formation in these conditions the thermotolerant coliform is confirmed [29]. The positive *Escherichia coli* tubes for thermotolerant coliforms are suspect for the presence of *E. coli*.

(4) Confirmation testing for *E. coli*: this method uses eosin methylene blue agar, which is a selective differential medium that distinguishes *E. coli* from other thermotolerant coliforms. If there is development of typical colonies of *E. coli* in this agar, these colonies are isolated for the biochemical proof of indole, methyl red, Voges-Proskauer and citrate (IMViC).

- *Sample preparation*: weigh 25 g of honey and add to 225 mL of peptone water 0.1% and homogenize the sample. This provides a 10^{-1} dilution; where 1 mL of the same corresponds to 0.1 g of the sample. A quantity of 1.0 mL of this solution (10^{-1}) is transferred using a new sterile pipette to a 9.0 mL of dilution water, thus obtaining a second decimal dilution (10^{-2}), where 1 mL corresponds to 0.01 g of the sample. In the same way, a 10^{-2} dilution provides a 10^{-3} solution (**Figure 1**).
- *Presumptive Test:* for presumptive evidence, 1 mL of the three subsequent dilutions should be inoculated in a series of three test tubes containing broth lauryl sulphate tryptose, with

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one series for each dilution. The tubes should be incubated at 35°C for 24–48 hours. If after this time there is turbidity of the medium and the formation of gas in the Durham tube, the presumptive test is positive for the presence of coliforms and should be subjected to confirmatory tests. If there is no turbidity in the medium or gas formation during the incubation period, the analysis ends at this stage and the test result is negative.

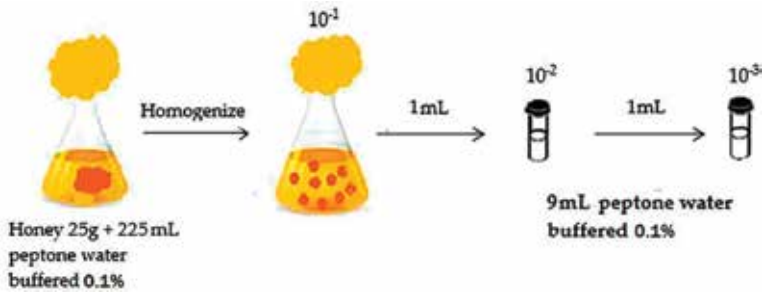

Figure 1. Decimal dilutions prepared (10^{-1}; 10^{-2} and 10^{-3}) from honey sample.

- *Confirmation test of total and thermotolerant coliforms:* to confirm the total coliform transfer, with a previously heated and cooled platinum inoculation loop, three loops from each positive tube and inoculate in a corresponding tube containing the bright green bile broth and incubate at 35°C for 24–48 hours. At the same time, perform a confirmatory test for the coliforms, similarly transferring the same ratios to tubes of *Escherichia coli* broth and incubating at 44.5°C for 24–48 hours. After this time, for the two tests, the formation of gas is observed in the Durham tube. If there is clouding of the medium and gas formation in the Durham tubes in the bright green bile broth the presence of total coliforms is confirmed, while the *Escherichia coli* broth confirms faecal coliforms. If there is no turbidity of the medium and no gas formation in the Durham pipes, the test is considered negative.
- *Biochemical test to confirm E. coli*: the tubes that present positive results for thermotolerant coliforms and/or tubes positive for coliform 35°C should be plated with a platinum loop with streaking on the surface of the Levine eosin methylene blue agar culture medium. The plates should be incubated at 35°C for 24 hours. Two colonies characteristics of *E. coli* (which are semi-nucleated with black centres and the presence or absence of metallic green brightness) must be isolated and subjected to biochemical tests of indole, methyl red, Voges-Proskauer and citrate [29]. The cultures with the profiles + + - - (biotype 1) or - + - - (biotype 2) are considered confirmed (**Figure 2**).

Reading of test using most probable number (MPN): the most probable number technique is based on the statistical probability related to the frequency and occurrence of the most probable positive results in terms of the real number of microorganisms present. Three sets of three tubes are inoculated, employing dilutions 0.1; 0.01 and 0.001 mL/g of honey. Thus, the number of tubes per series of three consecutive dilutions is three, giving a total of nine tubes. The number of microorganisms in the original sample is determined using the most probable number tables (**Tables 1** and **2**), according to the Brazilian Association of Technical Standards [30].

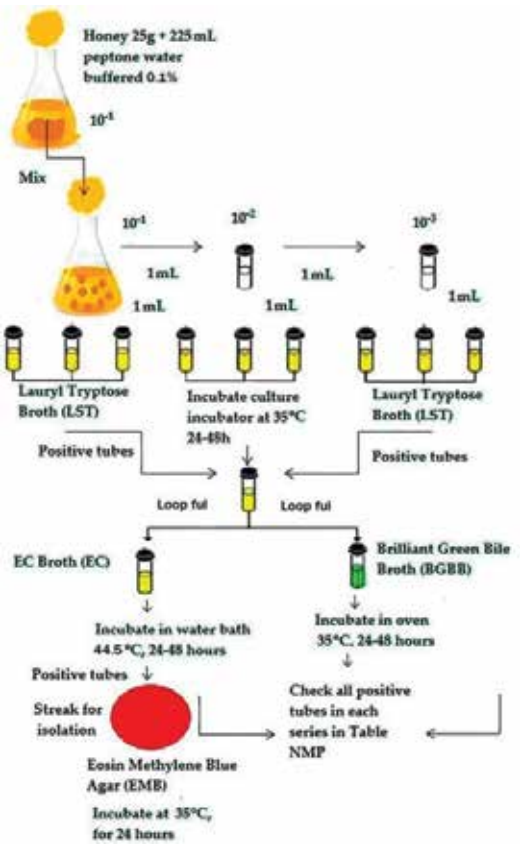

Figure 2. Presumptive and confirmatory tests of coliforms at 35 and 45°C.

Combination of positive tubes				Combination of positive tubes			
0.1g	**0.01g**	**0.001g**	**MPN**	**0.1g**	**0.01g**	**0.001g**	**MPN**
0	0	0	<3	2	0	0	9.1
0	0	1	3.0	2	0	1	14.0
0	0	2	6.0	2	0	2	20.0
0	0	3	9.0	2	0	3	26.0
0	1	0	3.0	2	1	0	15.0
0	1	1	6.1	2	1	1	20.0
0	1	2	9.2	2	1	2	27.0
0	1	3	12.0	2	1	3	34.0
0	2	0	6.2	2	2	0	21.0
0	2	1	9.3	2	2	1	28.0

Combination of positive tubes				Combination of positive tubes			
0.1g	**0.01g**	**0.001g**	**MPN**	**0.1g**	**0.01g**	**0.001g**	**MPN**
0	2	2	12.0	2	2	2	35.0
0	2	3	16.0	2	2	3	42.0
0	3	0	9.4	2	3	0	29.0
0	3	1	13.0	2	3	1	36.0
0	3	2	16.0	2	3	2	44.0
0	3	3	19.0	2	3	3	53.0
1	0	0	3.6	3	0	0	23.0
1	0	1	7.2	3	0	1	39.0
1	0	2	11.0	3	0	2	64.0
1	0	3	15.0	3	0	3	95.0
1	1	0	7.3	3	1	0	43.0
1	1	1	11.0	3	1	1	75.0
1	1	2	15.0	3	1	2	120.0
1	1	3	19.0	3	1	3	160.0
1	2	0	11.0	3	2	0	93.0
1	2	1	15.0	3	2	1	150.0
1	2	2	20.0	3	2	2	210.0
1	2	3	24.0	3	2	3	290.0
1	3	0	16.0	3	3	0	240.0
1	3	1	20.0	3	3	1	460.0
1	3	2	24.0	3	3	2	110.0
1	3	3	29.0	3	3	3	>1100.0

Source: ABNT MB-3463 [30].

Table 1. Most probable number (MPN) with 95% confidence limits for various combinations of positive results, using three tubes per series to inoculate 1 mL of dilutions 0.1; 0.01 and 0.001 g of honey/ml.

Example	Dilution (g/mL)			Tube combination	MPN/g
	0.1	**0.01**	**0.001**		
1	3/3	3/3	1/3	3,3,1	460
2	3/3	0/3	0/3	3,0,0	23
3	2/3	1/3	1/3	2,1,1	20

Table 2. Examples using dilution (g) combining 0.1; 0.01; 0.001 g/mL.

2.2. Yeast and mould counting

Counting of viable fungi is applicable to honey as it is an acidic food, with a pH of less than 4.5 and relatively low moisture. Fungi are affected little by variations in the pH range 3.0–8.0. The moulds grow below pH 2.0 and several yeasts below 1.5. When the pH deviates from the optimal, which is generally close to 5.0, the growth rate of colonies decreased and, if there are other inhibition factors, such as water or nutrient temperature activity, its restrictive effect on the growth rate becomes stronger [23].

Its presence at high levels in honey can provide various types of information; for example, the poor hygienic conditions of equipment, multiplying in the product due to failures in processing and/or storage. MERCOSUL GMC resolution n° 15 of 1994 approved the Technical Regulations for the Identity and Quality of honey, in view of resolutions n° 18 of 1992 and n °91 of 1993 of the Common Market Group [17], in which, in terms of hygiene, honey must be free of foreign inorganic or organic substances in its composition, such as insects, larvae and grains of sand, and should not exceed the maximum levels tolerable for microbiological contamination or toxic waste. Its preparation should be carried out according to the General Principles of Food Hygiene recommended by the Codex Alimentarius Commission—FAO/WHO [15]. In terms of fungi, up to 100 colony forming units per gram are tolerated in honey (CFU/g) [17].

Moulds are filamentous, multicellular fungi, and may be present in the soil, air, water and raw organic decomposition. They are generally aerobic and less demanding than other yeasts in terms of humidity, pH, temperature and nutrients. They can absorb any carbon source derived from food. As a nitrogen source, they can use nitrate, ammonia and organic nitrogen. They only grow on the surface of honey when in contact with air, as it is a food rich in carbohydrates and acids [23, 31].

Yeasts are classified as non-filamentous fungi whose form is unicellular and can be spherical, ovoid, cylindrical or triangular. They are usually spread by insect vectors and by wind and air currents [32]. For growth yeasts require moisture more than that required by moulds and less than that required by bacteria, with an ideal temperature range for growth at around 25 and 30°C. The growth of the osmophilic yeasts which are part of the micro-biota of importance of honey is favoured as the liquid substrate provides a greater opportunity for the development of anaerobic conditions, due to possessing the ideal acid pH for use in the fermentation by which the yeast is transformed into sugar, which is used as an energy source in alcohol, when the water activity value is at least 0.65 [31, 33]. According to Pitt and Hocking [34], most osmophilic yeasts are of the genus *Zygosaccharomyces*, including *Z. rouxi*, *Z. bailii* and *Z. bisporus*. To control these microorganisms in honey the application of good hygiene practices is required, and it must be ensured that water activity or moisture content is within acceptable limits [15]. When the honey extracted from the beehive has a lower water activity than 0.60 the multiplication of osmophilic yeast does not occur.

Method: based on the verification of the ability of these microorganisms to develop in a culture media with a pH around 3.5 and incubation temperature 25 ± 1°C. The use of acidified mediums selectively favours the growth of fungi, inhibiting most of the bacteria present in food [2].

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- *Plate preparation process*: dilute the potato dextrose agar (PDA) medium; cook in a water bath to 46–48°C; acidify the medium to pH 3.5 by adding 1.5 mL of tartaric acid 10% solution for each 100 mL of the medium; pour 15–20 mL into the plates; wait to solidify on a flat surface; identify the plates, before use, dry the semi-open plates in a kiln at 50°C for about 15 minutes or in a laminar flow exposing the surface for the time required for complete drying.
- *Sample preparation:* aseptically remove 25 g of the sample, open the packaging in an aseptic chamber, close to the flame of the Bunsen burner and taking care so that all the tools and utensils used are sterilized and flamed at the time of use.
- *Preparation of dilutions:* add 225 mL of peptone water 0.1% and mix, obtaining the first dilution (10^{-1}). For the second dilution (10^{-2}), transfer 10 mL of the first dilution to 90 mL of peptone water 0.1% and for the third dilution (10^{-3}), using the same procedure (**Figure 3**).

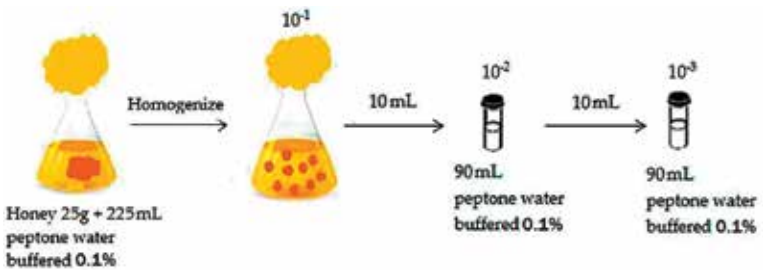

Figure 3. Procedure for the preparation of dilutions 10^{-1}; 10^{-2} and 10^{-3}.

- *Inoculation:* carry out surface plating, adding 0.1 mL of each dilution to the plates with the potato dextrose agar or dichloran-glycerol agar; with the help of Drigalski spatula or a hockey stick shaped rod spread the inoculum over the agar surface until its complete absorption.
- *Incubation:* incubate at 25°C for 5 days, without inverting the plates, in stacks of no more than three plates, in the dark. After incubation, check the presence of colonies of yeasts and moulds, count them and carry out the calculations (**Figure 4**).
- *Count the colonies and calculate results:* select the plates with 15–150 colonies with a colony counter. In the selected plate count and note separately the colonies with a filamentous appearance, characteristic of moulds. On the same plate count the other colonies, which can be yeast or bacteria, eventually capable of growth. Select at least five of these colonies and verify the morphology of the cells with a microscope observing if the culture is of yeasts, bacteria or a mixture of both. Colonies which present yeasts or mixtures of yeasts and bacteria are considered confirmed.

Determine the number of yeast colonies on the plate based on the confirmed percentage. For example, of 30 colonies counted, five were submitted to confirmation and three were confirmed as yeast (60%), so the number of yeast colonies on the plate is 30 × 0.6 = 18. To calculate the number of colony forming units per gram (CFU/g) of yeasts and moulds, multiply the number of colonies by ten and by the inverse of the dilution. The total calculation of both is

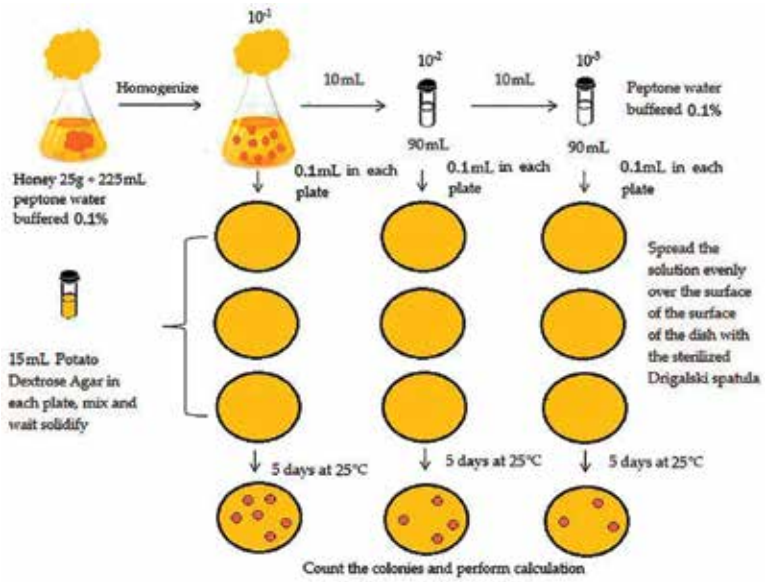

Figure 4. Procedure for the preparation and inoculation of dilutions 10^{-1}; 10^{-2} and 10^{-3} on the plates of potato dextrose agar.

carried out by adding the number of mould colonies and the number of colonies confirmed as yeast and multiplying by the inverse of the dilution according to Eq. (1).

$$\text{CFU/g} = \text{number of colonies x Dilution reverse x 10} \quad (1)$$

For example:
Dilution 10^{-2} (inoculated 0.1 mL)
Total typical colonies of mould on plate = 30
Presumptive colonies of yeast on plate = 40, five to submit for confirmation, confirmed four (80%)
Total yeast colonies on plate = 40 × 0.8 = 32
CFU/g moulds = $30 \times 10^2 \times 10 = 3.0 \times 10^4$
CFU/g yeast = $32 \times 10^2 \times 10 = 3.2 \times 10^4$
CFU/g of yeasts and moulds = $(30+32) \times 10^2 \times 10 = 6.2 \times 10^4$

2.3. Salmonella sp

Species of the *Salmonella* genus are agents of human and animal intestinal infections. Among the agents of foodborne illnesses, *Salmonella* is one of the most responsible for fatalities and clinical complications. Moreover, the high morbidity and mortality rate and incidence in humans and animals result in significant spending on medications and hospitalizations. The inspection and monitoring of food is aimed at the control and prevention of members of this group and the effects of their presence in food. Compliance with good manufacturing practices and control programs should include a certificate of compliance with the measures adopted, especially for this bacterial genus [23].

Method: the method for detecting *Salmonella* in food is based on its presence or absence, developed to guarantee detection even in unfavourable situations. The procedures recommended

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by various regulatory bodies basically follow five steps that can be applied to any type of food [2, 3, 5, 10, 11, 13].

- *Pre-enrichment in non-selective broth:* the objective is the recovery of injured cells, obtained by incubating the sample in non-selective conditions for at least 18 hours. The most commonly used medium is buffered peptone water and lactose broth. This step consists of aseptically weighing 25 g of honey in 225 mL of BPW 1% and incubating at 35°C for 18–24 hours. Finally, adjust the pH to 6.8–6.9 (**Figure 5**)

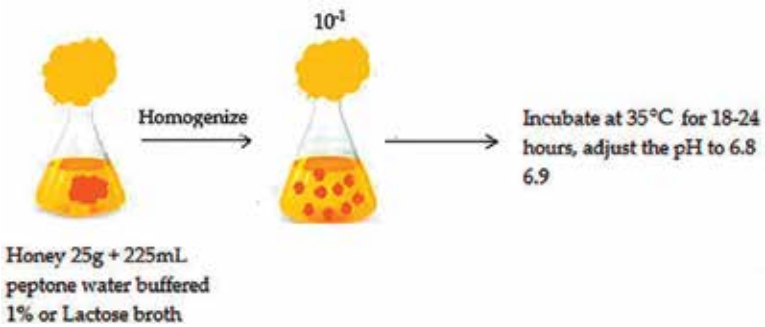

Figure 5. Procedure for the pre-enrichment non-selective broth.

- *Selective enrichment broth:* the objective is to inhibit the multiplication of the accompanying micro-biota and promoting the preferential increase of the number of *Salmonella* cells by incubating a pre-enriched sample in selective broth for 18–24 hours. The use of two different media is recommended because of the resistance of *Salmonella* to different selective agents of the medium which varies from strain to strain. The most recommended means are Rappaport-Vassiliadis soy broth (RVS) and selenite cystine broth (SC) as follows (**Figure 6**):

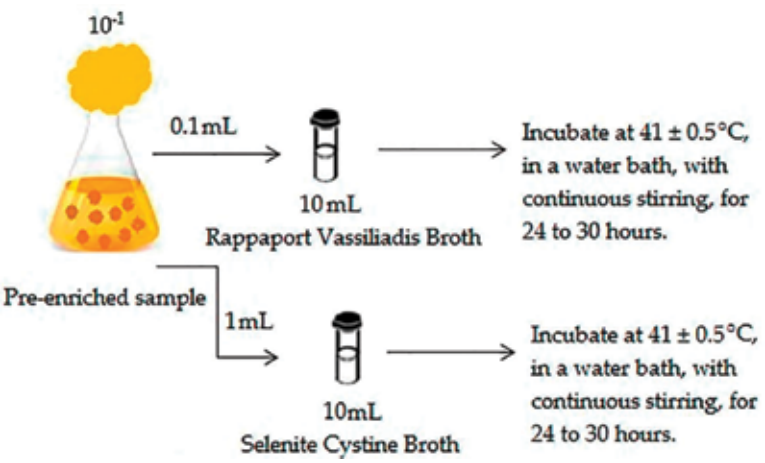

Figure 6. Procedure for the pre-enrichment selective broth.

- *Inoculation in Rappaport Vassiliadis broth:* pipette out 1 mL of pre-enriched samples and transfer to tubes containing 10 mL Rappaport Vassiliadis broth. Incubate the tubes at 41 ± 0.5°C in a water bath, preferably with continuous agitation or circulation of water, for 24–30 hours.
- *Inoculation in selenite cystine broth*: pipette out 1 mL of pre-enriched samples and transfer to tubes containing 10 mL of selenite cystine broth. Incubate the tubes at 41 ± 0.5°C in a water bath for 24–30 hours.
- *Selective differential plating*: the aim is to promote the preferential development of *Salmonella* colonies, whose typical characteristics differentiate them from competitors, for subsequent biochemical and serological confirmation. The use of more than one type of culture medium is recommended. The most commonly used media are those that differentiate *Salmonella* by the non-fermentation of lactose and by H_2S production, such as hektoen enteric agar (HE), xylose lysine deoxycholate agar (XLD) and xylose lysine tergitol-4 agar (XLT-4). As there are *Salmonella* strains which ferment lactose or do not produce H_2S, it is important that the second or third plating medium is not based on these characteristics. One option is the brilliant green phenol red lactose sucrose agar (BPLS) or brilliant green agar (BG) based on the fermentation of lactose but not the production of H_2S, and bismuth sulphite agar (BS), which is based on H_2S production and not lactose fermentation. Rambach agar can also be used in this step. Add 0.1 mL of novobiocin solution 4% to 100 mL of brilliant green phenol red lactose sucrose agar. Incubate all plates at 35°C for 24 hours (**Figure 7**).

Figure 7. Procedure for differential plating and biochemical identification.

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- *Isolation and selection*: depending on the medium, the *Salmonella* colonies present different colours after the incubation period: in hektoen enteric agar the colonies are green or bluish green, revealing or otherwise the production of hydrogen sulphide (H_2S) (dark centre); in xylose lysine deoxycholate agar the colonies are red with the production of hydrogen sulphide (H_2S) (dark centre); in xylose lysine tergitol-4 agar the colonies are red; in brilliant green phenol red lactose sucrose agar the colonies are colourless or have a pink colour, between clear and slightly opaque and when surrounded by fermenting microorganisms of lactose, may present a greenish-yellow colour. With brilliant green agar the colonies are red; and with bismuth sulphite agar (BS) the colonies are winged with a black centre.
- *Presumptive biochemical identification (screening methods*): the aim is to verify that the typical colonies obtained from the plates are truly *Salmonella*, specifically strains of *Salmonella enterica* subsp. *Enterica*, which is the main target for food analysis and which has a biochemical profile which is considered typical in detection assays (**Table 3**). Once the suggestive colonies have been selected by the indicating methods, they will be transferred to the screening mediums:

Culture medium used	Positive or negative reaction	Colour of culture medium	Positive or negative percentage
Glucose TSI (gas)	+	Yellow medium with gas	100.0
Glucose TSI (acid)	+	Yellow medium/red bevel	91.9
Lactose TSI	−	Red medium	99.2
Sucrose TSI	−	Red medium	99.5
TSI H_2S	+	Dark medium	91.6
LIA	+	Violet copper medium + H_2S production	98.0
SIM H_2S	+	Dark medium colour the base	97.0
SIM (indole)	−	No red ring	98.9
SIM (motility)	+	Diffusion in inoculation zone	97.0
Urea hydrolysis	−	Yellow medium	99.0
Lysine decarboxylase	+	Violet medium + H_2S	94.6
Ornithine decarboxylase	+	Violet medium + H_2S	97.0
Voges-Proskauer reaction	−	No red ring	100.0

Table 3. Colour of culture medium and *Salmonella* spp. positive and negative percentage in biochemistry test after 24 h incubated at 35°C.

(1) *Triple sugar iron agar (TSI Agar):* this medium is used to differentiate Gram-negative rods based on fermentation and the gas production from the carbohydrates: glucose, lactose and sucrose and the production of hydrogen sulphide. For the test, inoculate the triple sugar iron agar by deep, grooved stabbing motions and in inclined surface of the bevel. Incubate at 36°C

for 18–24 hours. In the presence of *Salmonella*, the glucose is rapidly depleted, and is verified by the appearance of a yellow colour in the base. After the fermentation of glucose, the aerobic degradation of the protein substrate of the medium occurs, producing ammonia, which gives the medium an alkaline pH, changing the bezel colouring to intense pink. Gas production is indicated by the formation of blisters or cracks in the medium. Most *Salmonellas* do not ferment sucrose and lactose. When these two sugars are not fermented, the apex keeps its original colour—amber. The production of H_2S is indicated by the black colour at the base of the central portion of the tube. Microorganisms such as *Proteus mirabilis, Edwardsiella tarda, C. freundii* and *Salmonella spp* may exhibit a similar behaviour.

(2) *Lysine-iron agar (LIA Agar)*: this medium is used to verify the decarboxylation of lysine which is evidenced by the violet colouration—alkaline—of the base. When this does not occur, the yellow colour indicates only the fermentation of glucose. The positive reaction for the deamination of lysine is visible at the apex (coppered violet) and the production of H_2S by the appearance of black colouring from the base to the central portion of the tube.

(3) *Hydrolysis of urea (Stuart urea broth and Christensen urea agar:* determines the ability of a microorganism to degrade, enzymatically, the urea by urease, with the formation of two molecules of ammonia and carbon dioxide, with the alkalization of the medium and increased pH. Streak only on the surface of the broth or the urea agar. Incubate at 36°C for 18–24 hours. The colour is caused by addition of the phenol red to the medium. The positive reaction turns the yellow (the original colour of the medium) to intense pink. *Proteus* features a more intense reaction; the negative reaction maintains the yellow colour of the medium. A total of 99% of the *Salmonella* strains do not produce urease.

(4) *Indole test (SIM medium):* check the motility of the microorganisms and the H_2S and indole production capacity. Inoculate the culture medium. Incubate at 36°C for 24–30 hours. The motility reading is characterized by the diffusion of growth throughout the medium. If restricted to the line of streaking, it indicates that the microorganism is immobile. After the motility reading, verify H_2S production by the development of the black colour in the medium. Bacteria that possess the tryptophanase enzyme are capable of hydrolyzing and deaminating the tryptophan with the production of indole, pyruvic acid and ammonia. To verify its production, add a few drops of Kovac's reactive to the tubes; if there is indole production a red ring will form. In most cases (99%) the strains of *Salmonella* do not produce indole, do produce H_2S and are mobile.

(5) *Voges-Proskauer (VP) test*: determine the ability of some bacteria to oxidize glucose producing organic acid as a final product. Transfer the microorganism to be tested to test tubes with red broth methyl-Voges-Proskauer (Clark and Lubs medium). Incubate the tubes at 37°C for 24–48 hours. To read, add 5 drops methyl red. Positive: red colour ($pH < 4.0$); Negative: original medium colour (yellow) ($pH \geq 6.0$). The *Salmonellas* are VP negative. From the VM-VP medium, remove 2 mL of culture to a new tube and add 15 drops of α-naphthol 5% reagent (reagent A) and 5 drops of KOH 40% solution (Reagent B) to each tube for each ml of culture medium. Agitate the tubes so that there is oxygenation of the medium. Wait for 10–30 minutes. Positive: development of pinkish to red colouring; Negative: absence of pink or red.

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(6) *Utilization of citrate (Simmons citrate agar):* characterize microorganisms capable of utilizing citrate as the sole carbon source, which cause the pH of the culture medium to increase due to the metabolism of citrate ions. Transfer by streaking the bacteria to be tested on the inclined surface of the Simmons citrate agar with a needle. Incubate the tubes at 37°C for 24–48 hours. The *Salmonella* strains (95%) are positive, except for the serotypes Typhi, Paratyphi A, Pullorum and Galinarum (100% of negative strains) and Choleraesuis (75% of negative strains), and can utilize the citrate and extract nitrogen ammonium salt, leading to alkalization of the medium from the conversion of the NH_3 in ammonia hydroxide (NH_4OH). After incubation, examine the cultures contained in the tubes with an inclined medium and assess the presence or absence of bacteria growth, checking for any change in colour: if positive, the medium becomes intense blue, especially at the apex; if negative, the natural colour of the medium does not change, but remains green.

(7) *Lysine decarboxylation:* determine the enzymatic ability of a microorganism by decarboxylating the amino acid lysine, with the subsequent alkalization of the medium, by the presence of the enzyme lysine decarboxylase. The colour is promoted by bromocresol (pupura indicator), which has a violet colour at alkaline pH. Inoculate with lysine iron agar with deep stabbing incisions, streaking the inclined surface of the bevel. Add sterile seal (Vaseline), to avoid the contact of the medium with air and the consequent appearance of a false alkalization on the surface of the medium by aerobically degrading the protein substrate. Incubate at 36°C for 24–30 hours. The majority of *Salmonellas* (96%) can produce lysine decarboxylase. During the initial period of incubation, the medium turns yellow due to the fermentation of glucose present. If the amino acid is decarboxylated, alkaline amines are formed and the colour of the medium returns to the original purple colour, with the production of H_2S.

(8) *Motility indole-8-ornithine (MIO) and motility indole-lysine (MIL):* inoculate by deep stabbing and incubate at 24 h/35°C. These mediums demonstrate the decarboxylation of ornithine or lysine amino acid, mobility and indole production. Mobility is interpreted by microorganism dissemination in the inoculation area (growth only in line of incision = negative motility); decarboxylation of ornithine or lysine is evidenced by a purple (alkaline) colouring in the base which neutralizes the acid (yellow) formed by the fermentation of glucose. Indole production is observed by the formation of a red ring after adding 2–4 drops of Kovacs reagent to the medium surface.

(9) *In malonate-phenylalanine broth*: determines the capacity of the microorganism to deaminate phenylalanine in phenylpyruvic acid, by its enzymatic activity, with consequent acidity. Inoculate the surface of the phenylalanine agar by streaking. Incubate at 36 ± 1°C for 18–24 hours. Add 2–3 drops of 10% ferric chloride solution. In a negative test, as there is no phenylpyruvic acid, the colour of the reactive FeCl3 remains yellow. The change of the colour on the bevel surface to green indicates the deamination reaction of the phenylalanine. *Salmonella* does not deaminate *phenylalanine*, with the colour of the medium remaining unchanged.

(10) *Dulcitol broth:* dulcitol fermentation occurs by turning the phenol red indicator to yellow. Most *Salmonella* are dulcitol positive (yellow).

Observations:

(1) These percentages indicate the incidence of strains with reactions marked as + or −.

(2) *S. Typhi* is anaerogenic;

(3) *Salmonella enterica arizonae*: + or – reaction to lactose, positive β-galactosidase;

(4) *Salmonella enterica salamae*: - reaction to lactose and β-galactosidase;

(5) *Salmonella pullorum* and *Salmonella gallinarum* are immobile;

(6) *S. arizonae* absorbs the malonate;

(7) *S. arizonae* does not ferment the dulcitol;

(8) 25% of the *Salmonella* strains are citrate-negative.

In general, the various regulatory bodies also recommend the use of miniaturized commercial kits which allow a great number of biochemical tests.

- *Serological test using fast agglutination*

Serologic confirmation verifies the presence of 'O', 'V' and 'H' antigens by agglutination tests with polyvalent antisera:

- Add approximately 2 mL of saline solution 0.85% to the culture in inclined nutrient agar and homogenize;
- With a Pasteur pipette deposit two drops of the suspension separately on a glass slide;
- Add one drop of anti-*Salmonella* polyvalent 'O' serum to one of the droplets of the suspension on the slide and mix, and add one drop of saline to the other;
- Perform the reading under illumination against a dark background for 1–2 minutes.

Classify the reaction as follows:

- Positive: presence of agglutination only in the cultivation + antiserum mixture.
- Negative: no agglutination in either mixture.
- Nonspecific: presence of agglutination in both mixtures (rough forms).

The cultures with positive results in the agglutination test with the anti-*Salmonella* polyvalent 'O' serum should be sent to certified laboratories for final classification.

2.4. Determination of the antibacterial activity of honey

With the exaggerated use of certain compounds such as ampicillin, cephalexin and others, bacteria have developed resistance to antibiotics, leading to studies for new compounds with

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antimicrobial activity from different natural products such as honey [35]. Since the beginning of civilization, honey has had a cultural importance that is not restricted merely to food but also includes use as folk medicine and as a cosmetic [36]. It has different therapeutic properties and is antimicrobial, antifungal, antioxidant, antiviral, anti-parasitic and anti-inflammatory [35, 37].

Honey is a substance prepared from the nectar of flowers (floral honey), plant exudates, or the excretion of sucking insects of plants (honeydew) [18]. The enzyme content present in honey is differentiated, as it depends on the species of bee, soil characteristics, seasonal factors such as temperature, rainfall and bee flora, with the product distinguished by the amount of organic acids, enzymes, vitamins, flavonoids, minerals and an extensive range of organic compounds, contributing to its colour, odour and specific flavour [38].

The antimicrobial action of honey is related to soil characteristics, atmospheric conditions, plant diversification, low water activity (Aw), high osmotic pressure, low pH, the glucose/oxidase system of hydrogen peroxide formation, the presence of phytochemical constituents and volatile substances [39]. These different qualities together create differences in the expression of antimicrobial activity of honey [40]. Molan [41] reported that in super-saturated sugar solutions, honey has a low water activity, which, as well as the natural acidification of the medium, creates unfavourable conditions for bacterial growth. In the presence of water and oxygen, the enzyme glucose oxidase converts glucose into gluconic acid and hydrogen peroxide, which are considered relevant substances for antioxidant action, which affect the microorganisms and preserve the sterility of honey during maturation [42].

Method: *Preparation of bacterial inoculum and standardization:* from the pure culture of bacteria preserved under refrigeration at 6°C, proceed to the preparation and standardization of the inoculum, in accordance with to the Clinical and Laboratory Standards Institute CLSI M07-A9 document [43]. Transfer three to five colonies of the selected strain to a test tube with a screw top containing 4–5 mL Miller Hinton broth (MHB), incubate the culture in the broth at 35°C for 18–24 hours and standardize the bacterial suspension in 0.85% saline solution, obtaining an optical turbidity comparable to standard McFarland solution 0.5 to the naked eye under illumination against a white background card with contrasting black lines. Dilute the inoculum at a ratio of 1:10 in saline solution 0.85% resulting in a concentration of 10^7 CFU/ml.

- *Antimicrobial susceptibility testing—broth microdilution method:* perform as per Clinical and Laboratory Standards Institute CLSI M07-A9 document [43], which is used to verify the minimum inhibitory concentration (MIC) by the broth microdilution method. To perform the test use 96 U bottom wells with markings indicating the position of each well, lines (A–H) and columns (1–12) (**Figure 8**).

Pipette out 100 μL of Mueller Hinton broth in each well and then perform a dilution series of different samples of honeys, with each honey sample in a different line. For serial dilution, pipette out 100 μL of honey in the first well, homogenize, remove 100 μl from the first well and transfer to the second well, remove 100 μL from the second and transfer to the third; and so on, until the ninth well of each row. This provides the following honey concentrations in percentages (%) (**Table 4**).

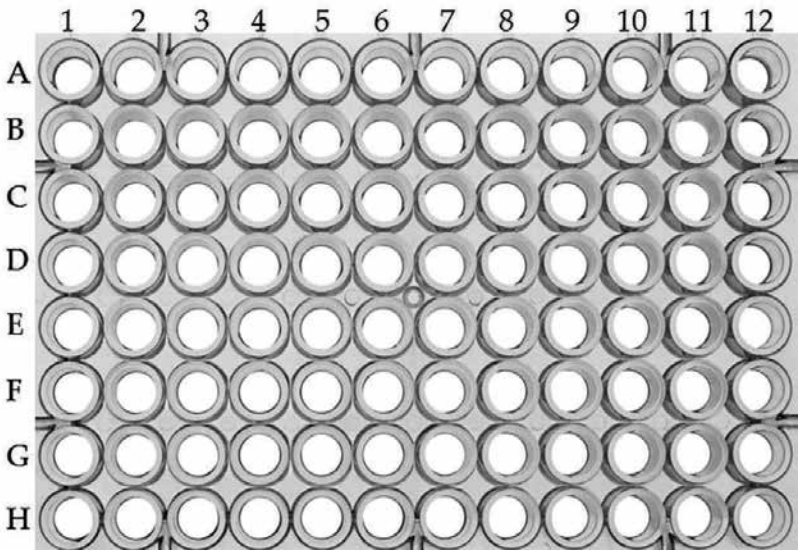

Figure 8. 96 well U bottom micro-plate with markings indicating the position of the lines (A–H) and columns (1–12).

No of wells	Honey (%)
1	50.000
2	25.000
3	12.500
4	6.250
5	3.125
6	1.560
7	0.780
8	0.390
9	0.195

Table 4. Honey concentrations in percentage (%) for wells from 1 to 9.

As a bacterial control (without the addition honey), use well 10, and as a broth control (without the addition of honey and inoculum), use well 11. After adding the honey, inoculate 5 μL of a standardized suspension of the bacteria in question in each well, except the broth control well, so that the end of test bacteria concentration is 5×10^4 CFU/well. Identify the micro-plates, incubate in a bacteriological incubator at 35°C for 24 hour. After 24 hours of incubation the micro-plates are analysed to determine the MIC, which is defined as the lowest concentration of honey in which there is no visible growth after incubation. Finally, analyse the well

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contents indicated with the minimum inhibitory concentration by microscope to confirm if there is growth or not. Perform the tests in triplicate for each of the bacteria analysed.

2.5. Microscopy of honey

Microscopy of food is a technique used to identify foreign components in products, making it possible to check if they comply with standards. Several countries use government and health-related agencies to ensure food safety, by monitoring their supply chains. MERCOSUR GMC Resolution N° 15/1994 approved the Technical Regulations for the Identity and Quality of Honey based on resolutions N°. 18/1992 and N° 91/1993 of the Common Market Group [17]. Normative Instruction N°. 11 of October 20, 2000 approved the Technical Regulations for the Identity and Quality of Honey [18] and pursuant to Ordinance N°. 46 of 10 February, 1998 the Ministry of Health and the Ministry of Agriculture and Supply established the adoption of the Hazard Analysis and Critical Control Point (HACCP) system by the Food Industries for animal products [44]. This system is recommended by international bodies such as the World Trade Organization (WTO) and the World Health Organization [16], both forming part of the United Nations (UN), for Food and Agriculture.

In compliance with these standards, any problem identified in honey lots should be corrected immediately and possible causes should be identified. Once the failures are identified, the company must take corrective action to prevent new problems arising. These corrective actions must be validated through audits and microbiological tests that prove the definitive correction of the non-compliance.

According to the macroscopic and microscopic criteria established in Brazil [18] honey must be free from any foreign substance. In practice, dirt present in honey may come from two sources—the first occurs inside the beehive, and is more difficult to control as it is added to honey by bees which carry fragments of other insects, pollen and soil. Secondary sources are present from harvesting through the steps of obtaining, processing, and distribution of honey [45], and include wax fragments, propolis, larvae, wood fragments and among others. Use of Good Apicultural Practices reduces the risk of secondary contamination ensuring a quality product in accordance with standard rules [46]. Camargo [47] recommends the procedures of the Good Apicultural Practices should be applied during the processing of honey, including: use stainless steel trays for stacking wooden beehives, allow no contact between the wooden beehive and the ground; choose honeycombs free of bees, larvae, or pollen; open wooden beehives only in the reception of the honey house for prior cleaning (removing of adhered bees, wax and propolis); filter the honey with the aid of sieves with meshes of various diameters, pumps or filters; decant the honey for soil removal at lower densities.

Method: The analysis of dirt and foreign matter can be performed following the method of the Association of Official Analytical Chemistry AOAC [9] N°. 945.79, which uses filtration of the sample in the presence of nitric acid. The method is based on dissolving 100 g of the honey sample in 200 mL of distilled water which is heated and acidified with 5 mL of nitric acid (HNO_3) at a concentration of 6 M. Filter the sample in a Buchner funnel. Mark four quadrants

on filter paper. Analyse using a stereoscopic microscope with a total multiplication of 100× and confirm the type of sediment between slide and cover slip under an optical microscope with a multiplication of 100–400×.

2.6. Clostridium botulinum

The pathogenic microorganism of importance in honey is the *Clostridium botulinum* bacterium, which is capable of producing spores. Bacterial spores are latent and resistant to adverse environmental conditions and can thus endure processing and storage for long periods. Contamination of honey by *C. botulinum* spores occurs within the colony, making practical procedures for its prevention difficult.

In practice, the bees carry the spores of this bacterium in their legs and antennae, taken from the soil where they land constantly. These spores begin to grow in the colonies, and remain in the combs together with the honey. Contamination is also possible in the act of collecting the product if hygiene practices are poor, and further contamination can occur through contact with the ground. Once present in honey, it survives in the medium without competition from other microorganisms. The incidence of spores in honey may also be related to multiplication and sporulation in dead bees and their larval forms in the colonies [48].

Honey is the only food recognized as a risk factor for infant botulism. Although there have been many cases of occurrence of infant botulism from honey contaminated with *Clostridium botulinum*, literature on this topic remains scarce. Consequently, in Brazil the administration of honey to children is not recommended, especially in the breastfeeding phase. This practice is also adopted in the United States, the United Kingdom and Argentina, where spores were isolated [49].

This disease occurs in children under 12 months, and 95% of cases occur in the first 6 months of life, when honey is used as a sweetener for bottles and juices as well as to bathe pacifiers to soothe the child. A child's intestine possesses an immature flora. The intake of honey with spores leads to germination, multiplication and the production of *botulinum* neurotoxins in the intestinal lumen, causing many problems for the health of children [50]. The consumption of honey by adults or older children does not seem to provide any kind of risk in relation to botulism. Consequently, it is recommended by the World Health Organization and the US Centers for Diseases that honey should not be given to infants under 6 and 12 months, respectively [16, 31].

Honey added as an ingredient in commercial infant formulas for babies aged less than 1 year must be thermally processed to destroy *botulinum* spores. No reports exist about the use of honey as an ingredient in other foods which have caused botulism. The analysis of honey for *C. botulinum* is not recommended as a control measure [49].

The microbiological analysis of honey detects product contamination. The presence of microorganisms or their spores in honey can cause its deterioration and result in enzymatic changes, the production of mycotoxins and even consumer illness. Due to the therapeutic properties attributed to honey, antimicrobial evaluation is essential to contribute to the quality maintenance of this product, adding to its commercial value.

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Chapter 13

Honey as a Functional Food

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Additional information is available at the end of the chapter

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Abstract

The most well-known functional properties of honey are its antioxidant and antimicrobial activities. The bioactive components of honey are affected by the flora from which it is produced and by geographical variations. Phenolic compounds promote, among other activities, high antioxidant action, being capable of minimizing intracellular oxidative damage associated with cellular aging, apoptosis and neurodegenerative diseases. A living cell system would provide a better platform for determining antioxidant activity, since the bioactive honey compounds can act modulating antioxidant defense gene expression. Indeed, phenolic compounds, amino acids and reducing sugars are among the substances responsible for honey antioxidant activity. Most of phenolic compounds also exert antimicrobial activity against a number of pathogens and spoilage microorganisms. The antimicrobial activity of honey is also due to the action of enzymes. In addition, honey was found to contain lactic acid bacteria (LAB), which itself produce a myriad of active compounds that remain in variable amounts in mature honey. In addition, these antioxidant compounds might play a key role as prebiotic, protecting and stimulating growth of probiotic bacteria. Oligosaccharides present in honey are well-known prebiotic substances stimulating growth, activity and protecting probiotic bacteria during passage through the gastrointestinal tract and during storage of the products. This chapter describes the main bioactive components of honey, especially with respect to the phenolic compounds and their antioxidant activity and assay methods.

Keywords: oligosaccharides, antioxidants, prebiotic

1. Introduction

Honey is a complex product that can be easily digested and assimilation and is produced from the nectar, a sugary liquid of flowers, due to action of bee enzymes (diastase, invertase and glucose oxidase) [1].

The great majority of the dry weight of honey (95–98%) consists of carbohydrates, mainly glucose and fructose, but also sucrose, maltose and other oligosaccharides. A minor portion (2–5%) is made up of various secondary metabolites, such as polyphenols and flavonoids, minerals, proteins, amino acids, enzymes, organic acids, minerals, vitamins, fatty acids, pollen and other solid particles from the process of obtaining honey [1, 2]. It also contains traces of fungi, algae, yeasts and lactic acid bacteria (LAB) [3].

Prebiotics are substances that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, the probiotic bacteria. Honey is often used as a sweetener but its use in medical preparations date from ancient cultures [4, 5]. Such functional properties includes antibacterial, antioxidant, antitumor, anti-inflammatory, antibrowning and antiviral [6, 7]. More recently, it was also found to be prebiotic and even a source of probiotic microorganisms [8, 9].

Antioxidant activity is defined as the capability of a compound to protect an organism from oxidant attack. Two widely used methods to verify this capability are the diphenylpicrylhydrazyl (DPPH) and the 2,2′-azinobis (3- ethylbenzthiazoline-6-sulfonic acid) (ABTS) assays. Both of them share the same mechanism of the reduction of the stable free radical but not measure the effect of an antioxidant on cell survival [9]. The biological yeast-based method can also measure the ability of a compound to induce cellular resistance to the damaging effects of oxidants [10, 11].

This chapter describes the main bioactive components of honey, with emphasis on phenolic compounds, antioxidant activity and assay methods.

2. Honeybee composition

Honeybees exist before human inhabits the Earth. It is formed due to action of honeybee's enzymes (diastase, invertase and glucose oxidase) on nectar or secretions of flowers. Honey is composed of various sugars, mainly glucose and fructose, but also sucrose, maltose and other oligosaccharides. In addition, honey contains proteins, amino acids, enzymes, organic acids, minerals and pollen. Besides, it can also contains traces of fungi, algae, yeasts and other solid particles from the process of obtaining honey [12] and lactic acid bacteria (LAB) [3].

Overall, honey contains acids, such as gluconic, succinic, malic, acetic, citric and butyric acid. Gluconic acid is found in greater amounts and is produced by action of glucose oxidase enzyme on the glucose to produce gluconic acid and hydrogen peroxide. Eighteen free amino acids occur in honey. Proline is the most abundant. Honey has small amount of vitamins that are negligible in the nutritional point of view. Therein includes ascorbic acid, niacin, pantothenic acid, riboflavin and thiamine. The minerals found in honey are potassium, sodium, calcium, magnesium, chlorine, iron, copper, manganese, phosphorus, sulfur and silica. Its content level of minerals is very variable and depends on the nectar source. Besides honey contains small amount of vegetable substances that contribute to the aroma and taste.

Honey has a set of five biologically active enzymes: Enzyme invertase (responsible for sucrose hydrolysis), diastase (which digests starch produced by plants), glucose oxidase (responsible for the production of acid and hydrogen peroxide), catalase (which uses hydrogen peroxide as substrate) and acid phosphatase. All these enzymes are derived from the glandular secretions of the honeybee. Hydroxymethylfurfural (HMF) can be found in low amounts in honey, which is produced by the decomposition of fructose in the presence of free acids, a process that occurs constantly in honey. The production of HMF depends on the temperature/time that the honey is subjected, particularly during pasteurization and storage [12].

3. Honey as probiotic source

Probiotic was originally defined by Parker [13] as "organisms and substances which contribute to intestinal balance." Later, Fuller [14] redefined as "viable microbial supplement which beneficially affects the host by improving the intestinal microbial balance, having specific effect in preventing pathological condition." Fuller's definition showed the need for the viability of probiotics in the food matrices and after passing the gastrointestinal tract. Probiotic definition has been expanded, not restricting to the health effects on the indigenous microbiota. According to Schaafsma [15], "oral Probiotics are microorganisms which upon ingestion in certain numbers, exert health effects beyond the inherent basic food nutrition."

The honey relationship with probiotic microorganisms is already in the generation of honeybees, when honeybees to be fed with honey over the 21 days of generation are stimulated immunologically due to probiotics contained in honey [16].

For a long time, researchers believed that the source of lactic acid bacteria in the honey was pollen and secretions of flowers that arrived to honey transported by honeybees. However, later studies proved that the lactic acid bacteria are present in the stomach of the honeybees; therefore, it is a source of lactic acid bacteria. The colonization mechanism is not fully clarified yet [8].

In the honey production process, the enzyme glucose oxidase is responsible for the transformation of the glucose in galacturonic acid. This causes the natural acidification of honey and therefore its preservation. Then, the majority of pathogenic and spoilage microorganisms are inhibited [12]. Due to honey acidity, yeasts and lactic acid bacteria are the predominant microorganisms. Among the lactic acid bacteria, there are probiotic microorganism, especially those belonging to *Lactobacillus* and *Bifidobacterium* genus.

Within the most isolated species of *Lactobacilli* genus are those belonging to the species *L. apis, L. insects, L. alvei, L. plantarum, L. pentosus, L. parabuchneri, L. kunkeei, L. kefiri* [17], and *Lactobacillus acidophilus*. Among *Bifidobacterium* genus, novel species were identified, *B. asteroids* and *B. coryneform* [8]. LAB symbionts within honeybees are responsible for many of the antibacterial and therapeutic properties of honey [3].

Olofsson et al. [3] reported that 13 lactic acid bacteria symbionts from the honey stomach of honeybees (*Apis mellifera*) were also found in large concentrations in fresh honey as well as having a wide spectrum of antimicrobial activity against various honeybee pathogens and bacteria and yeasts from flowers. According to these authors, many of the unknown healing and antimicrobial properties of honey are linked with these LAB symbionts. Every single member of the LAB microbiota of honeybees produces different bioactive metabolites. Organic acids were produced by all tested strains but in different amounts. Lactic, formic and acetic acids were produced as well as a wide variety of other interesting metabolites such as benzene and 2-heptanone and also putative lactic acid bacteria proteins in different honey types, suggesting their importance in honey production and antimicrobial activity.

4. Honey as prebiotic

The most well-known properties of honey are its antioxidant and antimicrobial contents. Different types of honey contain different characteristics and properties. Hence, the different sources of honey reflect its content and characteristics.

Prebiotics are substances that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, the probiotic bacteria. Traditionally, prebiotics were related to nondigestible oligosaccharides and polysaccharides substances, which beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the intestinal colon [18, 19]. However, this concept should be expanded to other substances, present in honey, which selectively benefit probiotic bacteria by stimulating is growth or activity. Most of the antioxidant compounds present in honey affect the viability of a series of undesirable microorganisms but does not affect probiotic bacteria or, in many cases, even stimulate their growth or activity [20–22].

Honey oligosaccharides had a potential prebiotic activity. These compounds selectively stimulate the growth of beneficial microorganisms, such as *Lactobacillus* and *Bifidobacterium* [23, 24]. Sanz et al. [24] conducted a study on how honey oligosaccharide affects the bacteria population in human gut intestinal track (GIT) and found honey that contain higher amount of oligosaccharide resulted in large amount of beneficial bacteria's growth.

The main oligosaccharides found in honeys surveyed in Brazil were the disaccharides, turanose, nigerose, melibiose, sucrose, isomaltose and four trisaccharides, maltotriose, panose, melezitose and raffinose [25]. Sanz et al. [24] found the highest amounts of maltulose and turanose (0.66–3.52 and 0.72–2.87 g/100 g of honey, respectively) in samples of honey from different regions of Spain and commercially available nectar and honeydew honeys. The trisaccharides, melezitose and panose, were the most abundant oligosaccharides from New Zealand honeys [26]. The fructooligosaccharides (FOS) quantified from wild Malaysian honeys were inulobiose, kestose and nystose [23].

Both lactobacilli and bifidobacteria are benefited in environments with low redox potential, and the presence of antioxidant compounds in honey is important in this regard. Flavonoids, amino acids and phenolic acids are the main antioxidant compounds in honey. Most valuable and superior antioxidant compounds of honey such as some phenolic compounds and glutathione are unstable over time and thermolabile. Thus, its final quality is compromised when raw honey goes through conventional thermal processing.

The main criteria for selection of probiotics are resistance to gastrointestinal conditions [14, 27]; characterization of genus, species, strain and its origin [27]; antimicrobial activity, adhesion to the intestinal epithelium, interaction between probiotics and intestinal microbiota of the host; absence of history of pathogenicity and infectivity; metabolic activity of bile salts; lack of hemolytic activity; absence of genes that convey resistance to antibiotics [28]; potential for reducing biofilm formation by pathogenic microorganisms and resistance to lysozyme besides technological properties [29]. As safety criteria, besides being nonpathogenic, the cultures must have no history of disease, do not deconjugate bile salts or produce toxins, shall not adduce antibiotic resistance genes and do not translocate or induce them, and preferably to be of human origin [27].

We studied the effect of adding 5% of honey to fermented milks on the survival of *Lactobacillus paracasei* and *Lactobacillus rhamnosus* of human origin (isolated from fecal samples of infants), after simulated gastrointestinal tract conditions. The resistance of the examined strains under conditions simulating the gastrointestinal tract was tested as previously described [30] and modified by adding lysozyme (100 μg/mL) to intestinal juice. The production process is shown in **Figure 1**.

Honey did not affect the survival of *L. paracasei* but avoid the reduction of *L. rhamnosus* number. Adding honey (5% w/v) in fermented milk positively affects the survival of *L. rhamnosus* during simulated gastrointestinal conditions. In the presence of honey, the population of *L. rhamnosus* after simulated intestinal condition was more than one log cycle higher than control without honey (**Figure 2**).

Similar response was observed with the commercial *L. casei*-01 (Christian Hansen), which was not affected by the presence of honey, differently of *Bifidobacterium* strains [20].

Bifidobacterium are more sensitive to acids than *Lactobacillus* genus. In fermented milk, *Bifidobacterium longum* was more sensitive than *Bifidobacterium brevi* during storage at 10, 20 and 30°C for 10 days. The same was observed with the pH reduction in smoothie yogurt, *B. brevi* was not affected, whereas *Bifidobacterium longum* lost viability during pH reduction from 6.5 to 3.8 [31].

Indeed, the honey has prebiotic effect by stimulating the growth and activity of probiotic bacteria. Besides, because of osmotic constitution and composition of the honey, it acts as protectant to the passage of probiotic bacteria throughout gastrointestinal tract. In fact honey has three functions related to probiotics aspects: it may contain probiotic microorganisms itself, prebiotic substances and protective function to probiotics during the transit by gastrointestinal conditions.

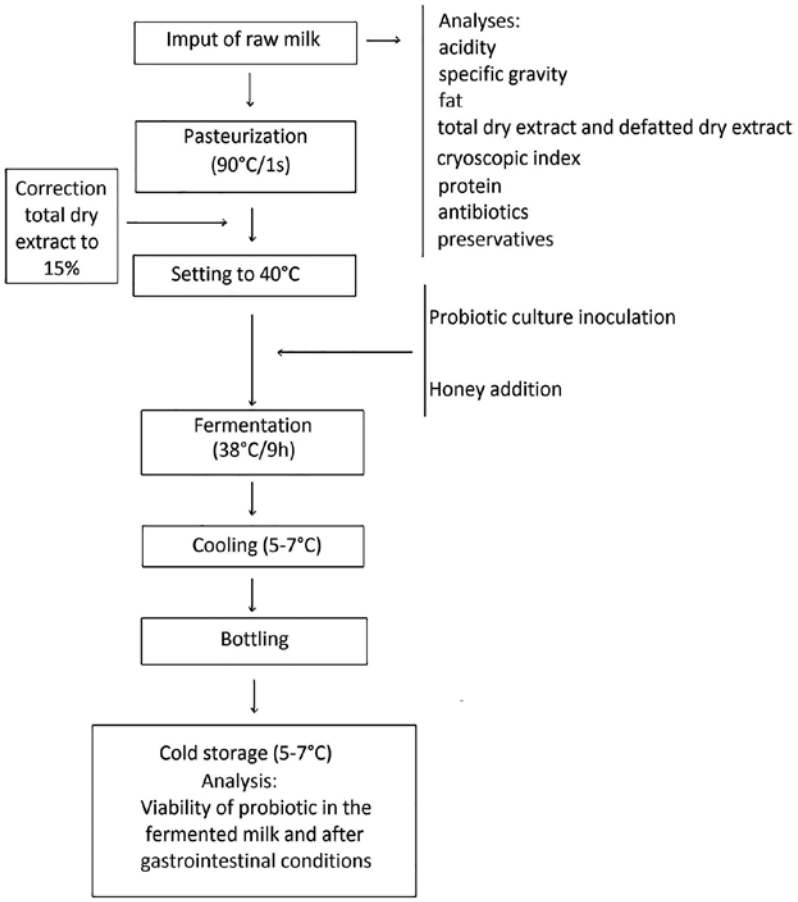

Figure 1. Production of probiotic fermented milk added with 5% of honey.

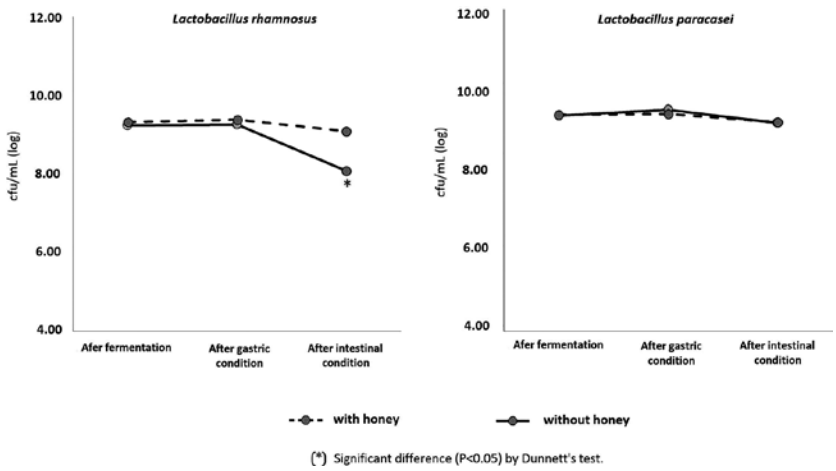

Figure 2. Survival of *Lactobacillus rhamnosus and Lactobacillus paracasei* after gastric and internal condition.

Favarin et al. [30] found that suspending free cells of two *Bifidobacterium* strains in honey solutions resulted in a protective effect, equivalent to the plain microencapsulation with sodium alginate 3% and concluded that microencapsulation and the addition of honey improved the ability of *Bifidobacterium* to tolerate gastrointestinal conditions *in vitro*.

5. Antioxidants of honey

During recent years, functional foods have attracted growing attention because of consumer's increasing concerns about their health, which has stimulated research effort into such foods [6]. An example, which emphasizes the importance of diet to health, is the French paradox, first observed in French population and found later also in other Mediterranean populations. Epidemiological studies revealed that antioxidant-rich diet is correlated with the increased longevity and decreased incidence of cardiovascular diseases observed in these populations despite their high fat diet, low exercise and smoking habits. It is well known that antioxidants can contribute to prevention of other illnesses, including neurodegenerative diseases, cancer and diabetes [32, 33].

Oxidative stress is an imbalance between oxidative and antioxidant molecules. The reactive species ($O_2^{\bullet-}$, $^{\bullet}OH$, H_2O_2 and others) have low stability and high reactivity resulting in low steady-state concentrations and high diversity of reactions they can participate in. Because of that, oxidative damage induces in biomolecules, as carbohydrates, proteins, lipids, and nucleic acids, which may alter its function, causing cells damage. As a consequence might flaw tissues and organs, leading to diseases [34]. Despite of their great capacity for damaging cells, other agents play important role, such as real players in many normal functions of living organisms, for instance in signalization of immune system cells [35].

Antioxidants are agents responsible for inhibition and reduction of injuries caused by reactive species in cell. Our genome encodes antioxidant enzymes to protect against oxidative damage, such as superoxide dismutase, catalase and glutathione peroxidase. Indeed, low molecular weight molecules as tocopherol, ascorbic acid and polyphenols can help on this process.

Free radicals can also affect food quality by reducing its nutritional content, color loss, unpleasant odors and flavors, promoting the development of food spoilage and, consequently, abbreviating their shelf life. Many synthetic antioxidants have been used in the food industries, but recent researches have mentioned their disadvantages and possible toxic properties for human and animal health [6, 34].

Honey and other bee products, whereby royal jelly and propolis may be used as functional foods because of their naturally high antioxidant potential, which could contributes to the prevention of certain illnesses [36–38]. Ancient Egyptians, Chinese, Greeks and Romans used honey in combination with vegetable or animal fat but also as part of all sorts of ointments [38]. The use of honey in modern medicine was strongly declined due to discovery of new drugs, but the search for more natural treatments boosts again search of honey and other products of bees [39].

Honey is a supersaturated solution of sugars (70–75%), of which fructose (38% w/w) and glucose (31% w/w) are the main contributors, 20–25% of water and about 3–5% for various substances [22, 38]. Hundreds of bioactive substances have already been found in honeys from different regions. This wide variation occurs when honeybees collect nectar from plants, incorporating secondary metabolites product of vegetables. This metabolism is rather variable and primarily depends on the botanical and geographical origin of the floral source, although certain external factors also play a role, such as seasonal and environmental factors and its processing [22, 40].

Honey antioxidant activity appeared to be a result of the combined effect of a range of compounds. Phenolic compounds (flavonoids and phenolic acids), as well as non-phenolic (ascorbic acid, carotenoid-like substances, organic and amino acids, and proteins including certain enzymes such as glucose oxidase and catalase) can contribute to honey antioxidant activity [40, 41].

The honey phenolic compounds are the main antioxidant compounds of honey. They are the phenolic acids and flavonoids, which are considered potential markers of the honey botanical origin. The phenolic acids are divided in two subclasses: the substituted benzoic acids and cinnamic acids. The flavonoids present in honey are divided into three classes with similar structure: flavonols, flavones and flavanones. These are important due to their contribution to honey color, taste and flavor and also due to their beneficial effects on health [21].

Large amount of research in honey also reports strong correlation between the total phenolic content and the antioxidant activity of honey extracts. Because of that, several literature reports have sought to identify and isolate them. Despite the relevant importance of polyphenolic compounds, which are recognized as the major constituents and responsible for the health-promoting properties of honey, their identification and quantification are of great interest for understanding their contributions to the overall bioactivity of honey [40].

6. Evaluation of the phenolic content

Analytical procedures used to determine polyphenols in a honey sample include their extraction from the matrix as well as their separation and quantification. The determination begins with an extraction step by means of solvents, which are mostly mixtures of water-alcohol in different proportions. Aqueous ethanol solutions (25–70 % v/v) are used in some work for 12–24 hours under stirring [42, 43]. While the methanolic extraction is used in different proportions with water [1, 44], there is still work using combined techniques of aqueous extraction, with heating or acidification, and subsequent ethanol extraction [40, 45]. Few studies conduct extraction with other solvents such as ethyl acetate [46].

The filtered or centrifuged extracts and different profiling techniques can be used for the determination of phenolic compounds. Liquid chromatography is considered to be the most useful separation technique for the analysis of polyphenols in different samples.

Coupled with various detection techniques, such as a diode array detector (DAD) [1, 21, 40, 47] and/or mass spectrometry, it enables both identification and quantification of polyphenols [42, 45, 46]. Since phenolic components can vary greatly, the suitable technique is liquid chromatography coupled with various types of mass detection, LC–MS enables high selectivity, sensitivity and universality when analyzing various polyphenolic components in their complex matrices.

Determination of a polyphenolic profile of honey is a complex task, so it is essential to develop separation and detection techniques, which would enable an unambiguous determination of as many components as possible. Tandem mass spectrometry is the detection method of choice when a comprehensive analysis of nontarget analyte is needed [46].

A wide variety of compounds isolated from honey and propolis come from flora, region and climate differences, where the nectar or sap was collected [12, 48, 49]. The phenolic compounds extracted, isolated and characterized can be classified into two major groups: phenolic acids and flavonoids.

The group of phenolic acids is divided into two main groups: derivatives of hydroxybenzoic acid (**Figure 3A**) and the hydroxycinnamic acid derivatives (**Figure 3B**). The benzoic acid derivatives include salicylic acid, gentistic, p-hydroxybenzoic, protocatechuic, vanillin, gallic, syringic and others. These are the most simple phenolic compounds found in foods [49, 50].

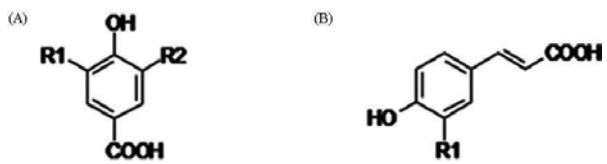

Figure 3. (A) General structure of derivatives of benzoic acid (benzoic acid, R1 = R2 = H). (B) General structure of the derivatives of hydroxycinnamic acid (hydroxycinnamic acid R1 = H) [12].

Hydroxycinnamic acid derivatives include p-coumaric, caffeic, ferulic, among others. They may also be in conjugated form between themselves or with other organic compounds. This is the case of chlorogenic acid, which is the combination of quinic acid and caffeic acid [49, 50]. All cited phenolic acids have been described in honey samples in different concentrations according to the flora collected by honeybees [40, 46].

Flavonoids are compounds that possess the diphenylpropane skeleton: two benzene rings linked through oxygen containing a pyran or pyrone ring [46] (**Figure 4**). Flavonoids are a group of substances comprising classes of flavonols, flavones, flavonones, isoflavones, anthocyanins and catechins. In plants, flavonoids are involved in pigmentation of fruits and flowers and the regulation of plant growth and plant protection against oxidative agents [32, 52]. In samples of honey and propolis naringenin, chrysin, rutin, morin, kaempferol, myricetin, hesperidin, apigenin, among others [40, 45, 46, 51] are found.

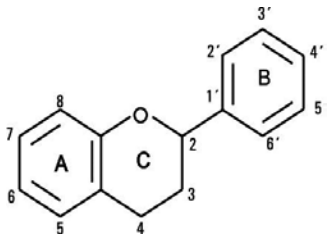

Figure 4. Structure of the major chemical types of flavonoids [51].

7. Phenolic profile of honey

Regions characterized by a hot and humid climate with very high exposure to sunlight (as in northeast Brazil) are particularly known to exert a marked influence on the polyphenolic content of plants. Sun-exposed plants such as juazeiro (*Ziziphus joazeiro* Mart.) can contain much more total phenolics than the same varieties or other when grown in the shady locations [53].

Assays made with honey collected in the central and southern region of Amazonas state in Brazil found that total phenolic content of methanolic extracts from the honey samples ranged from 17.0 to 66.0 mg galic acid equivalent (GAE)/g of extract and also high antioxidant profile. Gallic, 3,4-dihydroxybenzoic, 4-hydroxybenzoic, vanillic, salicylic, syringic, coumaric, trans,trans-abscisic, cis,trans-abscisic and cinnamic acids, catechol and flavonoids, taxifolin, naringenin and luteolin were identified. Concentrations ranged from 0.02 to 67.0 mg/mL of extracts, variating with the sample [54].

Brazilian honeys from the semiarid region, which were composed of 24 monofloral honeys produced by *Meliponini*, native species of bee, were found to present strong antioxidant activity. The total phenolic content varied from 0.31 to 1.26 mg GAE/g with differences ($p \leq 0.05$) among samples from distinct floral sources. The scavenging activity of DPPH radicals varied from 11.2 ± 1.3% to 46.9 ± 1.9%. Phenolic compounds p-coumaric, ellagic and 3,4-hydroxybenzoic acid and the flavonoids rutin, catechin, chrysin and naringenin were detected in higher amounts in *Ziziphus joazeiro* Mart. honeys than in the other honeys produced by the same bee species [40].

Fifty eight polyfloral honey samples, from different regions in Serbia, were studied to determine their phenolic profile, total phenolic content and antioxidant capacity. It was reported that the phenolic content ranged from 0.03 to 1.39 mg GAE/g and the radical scavenging activity of DPPH radicals ranged from 1.31 to 25.61% [44], an antioxidant capacity lower than that found in honey from high sunlight incidence regions.

All these studies found strong correlation between total phenolic content or total flavonoid content and radical inhibition capacity, indicating that phenolics and flavonoids are the primary factors responsible for the antioxidant properties of the studied honeys. Consequently, these results reinforce the influence of the botanical source on honey antioxidant properties.

Honey phenolic composition is not predictable, since it is highly related with the flora where honeybees collected nectar. Thus, the profile of phenolic compounds can be used to determine honey flora origin. For instance, a study in honeys produced in arid regions in northeast Brazil showed a high quantity of rutin in honeys from *Ziziphus spina-christi*, suggesting that it is a marker in honeys from the *Ziziphus* species [40]. Samples originated from Vojvodina and Zlatibor regions were clearly distinguished from those from the rest of Serbia because of the presence of dicaffeoylquinic acid, ellagic acid, caffeic acid phenethyl ester and chlorogenic acid, among others [45].

8. Mechanisms of action of phenolic compounds

Several mechanisms have been proposed to explain the observed antioxidant activity of phenolic compounds. The first is the direct removal of radicals through the formation of more stable compounds from radical supply of hydrogen (**Figure 5**). The various possible resonance hybrids in flavonoids and phenolic acids structure make them less reactive, limiting the deleterious power of other reactive species [55].

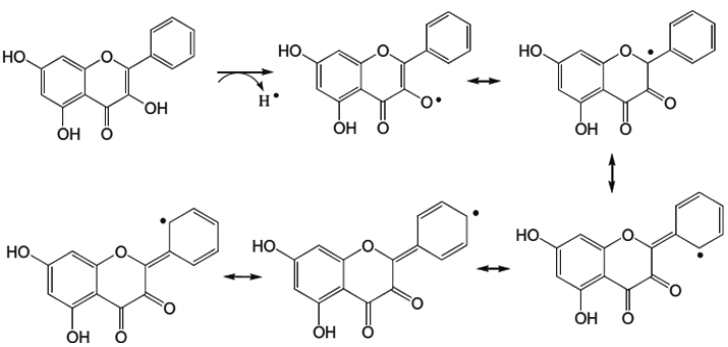

Figure 5. Radical stabilizing resonance structures by monoelectric oxidation of hydroxyl group in galangin [55].

Another mechanism of action of its antioxidant activity is their metal chelating propriety (**Figure 6**), which removes ions such as Fe^{2+}, which catalyzes the formation of free radicals by Fenton and Waber-Heiss reactions and which are propagators responsible by reactive oxygen species; decreasing, so the intracellular oxidative stress [56].

The *in vitro* activity of phenolic compounds depends on their structure. In flavonoids, the hydroxyl groups are in the ortho position (**Figure 7A**), especially in ring B; the presence of double bond to oxygen in the ring C (**Figure 7B**) and hydroxyl groups at positions 3 and 5 (**Figure 7C**) were found to increase the antioxidant capacity, since they contribute to stabilizing resonance structures [32, 57]. The presence of glycosides, however, reduces the antioxidant activity. The antioxidant activity of glycosidated conjugate rutin decrease about 50% when compared to quercetin [32].

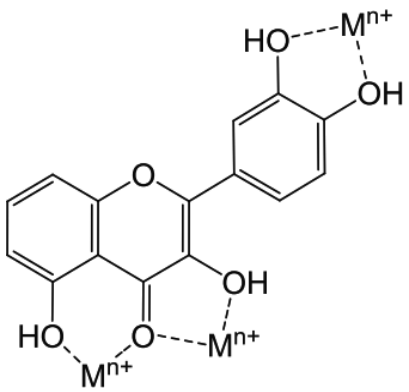

Figure 6. Possible flavonoid coordinating points with metals [32].

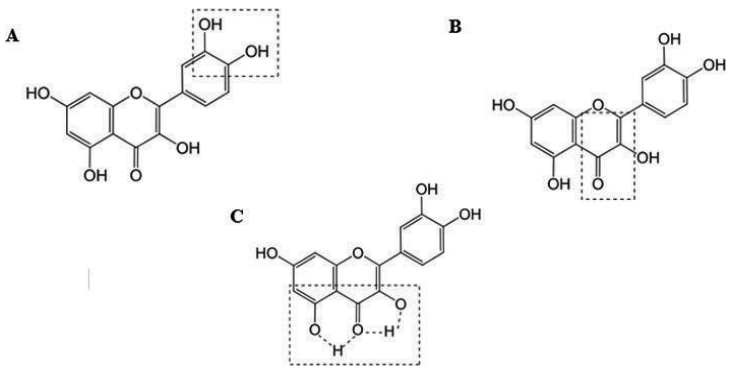

Figure 7. (A) Hydroxyl ortho position; (B) the presence of double-bonded oxygen in the 5-position of ring C; (C) the presence of hydroxyl at positions 3 and 5 [32].

Phenolic acids have increased activity in the presence of hydroxyl groups in the ortho position (**Figure 8**) or carbonyl groups in the ortho hydroxyls, as with syringic acid [57]. Moreover, in general, the hydroxycinnamic acids have shown *in vitro* activities higher than the hydroxybenzoic acids [58].

However, tests on biological models show that the flavonoids and other phenolic compounds act modulating the expression and activity of enzymes related to antioxidant defences [59, 60]. Phenolic compounds have the ability to induce phase II enzymes, such as quinone reductase NADPH and GST, as well as inhibiting enzymes related to carcinogenesis such as protein activation 1 (AP1), nuclear factor (NF)-κB and MAP-kinases [32, 60].

It is also important to emphasize that phenolic compounds also have pro-oxidant activity, dependent on its concentration. The presence of hydroxyl groups in the ortho position can also produce radicals or hydrogen peroxide, in the presence of copper ions and oxygen

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molecules [61, 62]. The flavonoid rutin and morin at concentrations above 100 μg mL^{-1} were able to produce hydrogen peroxide and damage DNA through comet assay in human lymphocytes. However, this effect was not observed with naringenin, and hesperidin in the same concentration, which do not have hydroxyl groups in ortho position on ring B [63]. The generation mechanism of hydrogen peroxide or radicals can explain the antimicrobial action of flavonoids and their toxic effects at higher concentrations to microorganisms [32].

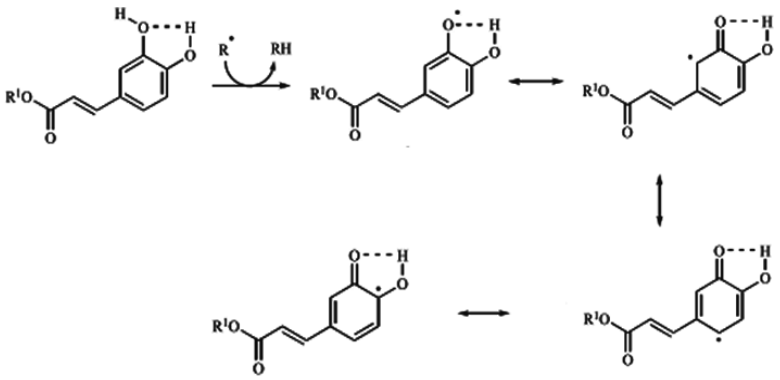

Figure 8. Radical sequestration mechanism of hydroxycinnamic acid including resonance stabilization radical by intramolecular hydrogen bonding [58].

In vivo testing confirms the antioxidant activity observed *in vitro*. Phenolic extracts from two monofloral Cuban honeys were able to inhibit erythrocytes oxidative damage. This study indicated that honey contains relevant antioxidant compounds responsible, at least in part, for its biological activity and that uptake of its flavonoids may provide defence and promote cell functions in erythrocytes [64]

A study was undertaken to determine whether replacing sucrose in the long-term diet with honey, which has high antioxidant content, could decrease deterioration in brain function during ageing. Rats were fed *ad libitum* for 52 weeks on a powdered diet that was either sugar-free or contained 7.9% sucrose or 10% honey. Apparently, long-term feeding of honey, sucrose and a sugar-free diet may have some effects on anxiety and spatial memory in rats, with honey-fed rats exhibiting less reduction in spatial memory and decreased anxiety at the completion of the study [65].

Manuka honey, derived from the *Leptospermum scoparium* tree, was investigated about its protection effect against oxidative damage and improvement of the process of skin wound healing, using human dermal fibroblast cells. Up to 16 compounds were identified in this honey, with leptosperin derivatives and methyl syringate as the major ones. It protected against apoptosis, intracellular ROS production and lipid and protein oxidative damage. Manuka honey also protected mitochondrial functionality, promoted cell proliferation and activated the AMPK/Nrf2 signaling pathway, associated with antioxidant defence, as well as the expression of the antioxidant enzymes such as SOD and CAT [37].

9. Antioxidant activity assays

Various assays have been applied to determine honey antioxidant activity. The most common ones are colorimetric assays, DPPH (1,1 diphenyl-2-picrylhydrazyl), ABTS (2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid)), FRAP (ferric reducing antioxidant power) and TEAC (Trolox equivalent antioxidant capacity), based on electron transfer, and ORAC (oxygen radical absorbance capacity) assay, based on hydrogen atom transfer and other techniques as voltammetric assays [34, 41, 46]. The total phenolic content is commonly spectrophotometrically determined with a Folin-Ciocalteu method, sometimes with modification and total flavonoid contents is generally measured by colorimetric assay with aluminum chloride [40, 54].

At the present time, no single available assay for testing the antioxidant capacity provides all the desired information. An evaluation of the overall antioxidant capacity may require multiple assays to generate an "antioxidant profile" encompassing reactivity towards both aqueous (DPPH and ABTS) and lipid/organic radicals (ORAC) directly through radical quenching and radical-reducing mechanisms (DPPH, ABTS, FRAP and ORAC) and indirectly through metal complexing (FRAP) [64].

Gorjanović et al. [41] evaluate hydrogen peroxide sequestration capacity of single bioactive compounds isolated from honey by voltammetric technique. As result, the flavonoids showed the highest hydrogen peroxide scavenging activity among the compounds, followed by phenolic acids. Activity of predominant honey sugars, fructose, glucose and maltose was found to be three orders of magnitude lower than tested flavonoids, but their contribution to total activity is significant due to their quantity. High hydrogen peroxide scavenging activity has been attributed to some amino acids, aromatic and basic ones, whereas non-polar amino acids, such as proline, the most prevalent amino acid in honey (0.40–2.2 mg/kg), possess low activity. Although phenolics are minor honey constituents, their antioxidant activity is high enough to correlate between honey hydrogen peroxide scavenge and total phenolic content [41].

Antioxidants *in vitro* assays do not consider physiological conditions such as concentration of intracellular metabolites nor does consider metabolic factors such as bioavailability and enzymatic transformations [58]. The *in vivo* assay, using yeast cells, specifically the specie *Saccharomyces cerevisiae,* represents an alternative to evaluate antioxidant activity. Yeasts are unicellular eukaryotic organisms widely studied and have great similarity with higher mammalian cells, especially in regard to the antioxidant defence system [66]. Because of this, it becomes an interesting biological model to evaluate biological activity related to natural extracts and molecules [66].

The use of *S. cerevisiae* cells as a study model has other important advantages. Its genome is completely elucidated, thereby facilitating the production of genetically modified strains for further studies; adding to this, its low cost of cells maintenance, ease of handling in the laboratory, rapid growth and low rate of spontaneous mutations [67]. Moreover, preliminary studies in yeast substituting the use of guinea pigs, rats and mice, certainly speeds up research work.

Furthermore, the *in vivo* assays measure the effect of an antioxidant on cell survival [9]. The biological yeast-based method can also measure the ability of a compound to induce cellular resistance to the damaging effects of oxidants [10, 11]. The determination of the lipid membrane integrity is an important parameter in verifying oxidative damage.

Lipid membrane peroxidation constitutes a primary cytotoxic event that triggers a sequence of lesions in the cell. Changes in membranes lead to disorders related to membrane permeability by changing the ionic flow and the flow of other substances, which results in the loss of selectivity for intake and/or outtake of nutrients and toxic substances to the cell, DNA damage and changes in the cell cycle [68, 69]. The Thiobarbituric Acid Reactive Substances (TBARS) assay method [70] measures the extent of lipid degradation by quantifying malondialdehyde (MDA) formed from the oxidation of triacylglycerols. In this method, the reagent thiobarbituric acid generates adduct with malondialdehyde, which is detectable spectrophotometrically at 532 nm. Besides the aforementioned method, cell viability assays are also employed in assessing oxidative damage in yeast, which evaluates the stress tolerance increase caused by treatment with antioxidant compounds [71]; mitochondrial function assays, since many apoptotic processes start in this organelle [72]; measurement of intracellular reactive oxygen species formation, using 2,7-dichlorofluorescein as indicator [71, 73]; protein carbonylation tests [74, 75], which is also formed as consequence of oxidative damage; assessment of energetic metabolism and enzymatic activity associated with the stress response [67, 74], among other methods.

Propolis, as well as honey, is a product of bees derived from the collection of plant fluids and alike contains phenolic compounds in its composition. Sá et al. [76] evaluated the antioxidant capacity of propolis extracts using a wild-type (BY4741) *S. cerevisiae* and antioxidant-deficient strains (Δctt1, Δsod1, Δgsh1, Δgtt1 and Δgtt2), either to 15 mM menadione or to 2 mM hydrogen peroxide during 60 min. They observed that all strains, except the mutant Δsod1, acquired tolerance when previously treated with 25 μg/mL of alcoholic propolis extract. Such a treatment reduced the levels of ROS generation and lipid peroxidation, after oxidative stress. However, cells were drastically affected by direct exposure to H_2O_2, after propolis treatment, survival increased almost three times. The increase in Cu/Zn-Sod activity by propolis suggests that the protection might be acting synergistically with Cu/Zn-Sod.

The antioxidative activities of propolis and its main phenolic compounds, caffeic acid, p-coumaric acid, ferulic acid and caffeic acid phenethyl ester (all 0.05 g/L), were investigated in the yeast *S. cerevisiae.* After 1 h of yeast cells exposure, their intracellular oxidation was measured using 2,7-dichlorofluorescein. Yeast cells exposed to 96% ethanolic extracts of propolis in DMSO showed 42% decreased intracellular oxidation compared with nontreated cells, with no significant differences seen for the individual phenolic compounds [72].

It is concluded that honey and other bee products possess proven *in vivo* and *in vitro* antioxidant activity, and this property can be the foundation of the functional properties assigned to them.

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Section 2

Detection of Residues and Heavy Metals

Chapter 14

Analytical Procedures for Determining Heavy Metal Contents in Honey: A Bioindicator of Environmental Pollution

Enrique Mejías and Tatiana Garrido

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/66328

Abstract

Metals are pollutant residues detectable in honey and in fact account for most of the inorganic pollutants found in this food product. Metal pollutants can be accumulated through the food chain and, at levels exceeding safe thresholds, can be toxic to humans and even damage physiological functions. During the honey-making process, bees can transport pollutants to the beehive following contact with polluted botanic species or from drinking contaminated water. Detecting very low concentrations is a persisting challenge to accurately measure these elements in honey. Additionally, since honey is a complex organic matrix, treatments are needed prior to applying any classical chemical methods for metal determination, such as inductively coupled plasma and atomic absorption spectroscopy. Therefore, optimal results are dependent on adequate sample conditioning prior to heavy metal content analyses. Chemical pretreatments include calcination processes and/or acid digestion. Regarding execution, the last steps of any metal detection methodology are the primary determinants of result quality, where any loss of mass is reflected by unreliable values.

Keywords: heavy metals, metals in honey, sample preparation, analytical methods

1. Introduction

The internationally recognized Codex Alimentarius Commission defines honey as a naturally sweet substance produced by bees through the collection of flower nectar or secretions from living plants and the subsequent transformation of these collected materials with substances inherent to bees. This mix is deposited and dehydrated for storage, a process that results in the maturation of honey [1].

Honey is principally composed of a complex mix of carbohydrates, among which fructose and glucose account for 85–95% of the total sugars. Since glucose is less soluble than fructose, the proportions of these sugars in honey determine overall granulation, with higher fructose quantities lending to honey that remains longer in the liquid state [2, 3]. Other more complex sugars are formed through the bonding of two or more fructose/glucose molecules with trace polysaccharide residues. Honey also contains other substances to lesser degrees, including organic acids, amino acids, proteins, enzymes, minerals, fat-soluble vitamins, flavonoids with antioxidant properties, and hydroxymethylfurfural, a compound that indicates honey freshness [4–8]. Finally, honey can be further classified by melissopalynological analysis as either monofloral or polyfloral in origin. Monofloral honey is of greater commercial value due to 45% of solid residues being single-pollen in origin [9–12]. Altogether, the quality of honey depends on the presence and concentrations for each of the aforementioned compounds, as well as on classification as either mono- or polyfloral.

The close source-product association between plants-honey means that all honey inherit various characteristics of and share biological properties with their respective botanic sources [13]. Due to this, undesirable compounds or residues can be found in honey if the source plants were exposed to these substances, including those of anthrophic origin. Among the residues that alter the natural composition of honey are metals, which, depending on their concentration in food, can pose as a human health risk [14]. The most common route through which humans ingest and are exposed to metals is through the diet, although the presence of these chemical elements in the air also means intake through inhalation.

Some heavy metals are essential elements for normal growth of plants such as Co, Fe, Mn, Ni, Zn, and Cu and they have important roles in metabolism, but at higher concentrations, the same metals become toxic. Those increased levels can cause a decrease in percentage of biomass in vegetables and in many other cases, they lead to plant death. On the contrary, some heavy metals such as Pb, `Cd, Cr, and Hg have been marked with high toxicity for plants [15].

Metals have a density, (d) > 5 g/mL and atomic number > 20, with the exceptions of alkaline and alkaline earth metals. No more than 0.1% of the earth's crust contains metals. Although the term "heavy metals" primarily refers to elements with elevated cellular toxicity, this definition now extends to include micronutrients that, at high concentrations, represent a risk to human health. Heavy metals without known biological functions are the most dangerous due to high toxicities, including barium (Ba), cadmium (Cd), mercury (Hg), lead (Pb), strontium (Sr), and bismuth (Bi). Trace elements, or micronutrients, toxic at increased concentrations include boron (B), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), magnesium (Mn), molybdenum (Mo), nickel (Ni), selenium (Se), and zinc (Zn) [16]. Due to the human health risk presented by these heavy metals and micronutrients, regulations exist for the maximum residual limits permitted in various foods destined for human consumption [17, 18].

2. Metals in soils: impacts on apiculture

The origin of heavy metals in soils can be anthropic or natural, and may be associated with different fractions of soil, which determine the mobility and availability of these metals to

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the surrounding ecosystem. It could affect honeybees or its habitat by polluting plants and water. The availability and mobility of these contaminants could be modified in relation to the physicochemical properties of the soil, for example, pH and organic matter content, among others.

Undoubtedly, this will have an important effect in the metal content in honey, since honeybees are able to take water from these polluted sources. Moreover, bees may transport these pollutants to beehives by fixing them to their bodies after their contact with the polluted plant species.

Soils systems are complex and vary in traits based on mineral and organic residue compositions. In particular, heavy metals of both anthrophic and geochemical origins can affect soil characteristics. For example, Chile is the leading producer of copper worldwide, with this metal constituting a primary source of both net national income and employment. Nevertheless, copper mines generally overlap with the Transverse Valleys of the "Norte Chico" region of Chile, which is also an important region for agriculture and apiculture. Due to this spatial crossover, controversies exist between the mining and apiculture industries regarding mining-produced wastes. Specifically, these toxic residues are discharged into the air, soil, and water of valleys with human populations and with ranch, farm, and apiculture productions.

The presence of heavy metals in soils is not only due to external contamination, but can also be of geochemical in origin. Indeed, high copper contents can be found internationally in a number of soils [19]. This can occur due to mixed causes, such as abnormal native geochemical contents being complemented by mining contaminants. Generally, copper contamination is accompanied by high contents of other metals, such as arsenic, lead, cadmium, and zinc. Soils are open systems that exchange energy and organic matter with proximal environments. These exchanges are typified by a heterogeneous mix of three principal components—solid, gaseous, and liquid fractions of organic, inorganic, and microorganic components [20]. Several analytical approaches exist for determining total heavy metal contents or the fraction of total soil content represented by these elements. This fraction can be used to determine metal availability and mobility. Element availability in soils is the most representative way to estimate total element content as this fraction facilitates establishing assumptions of mobility, plant absorption, and possible contamination [21]. The availability of distinct contaminating elements depends on properties inherent to each element, including the tendency to form complexes with organic material; mineral chemisorption; precipitation as insoluble sulfides, carbonates, phosphates, and oxides; and co-precipitation in other minerals [22].

One of the most important chemical processes in soils is adsorption. This process determines the quantities of nutrients, metals, pesticides, and other organic chemical components retained on the soil surface. Due to these functions, adsorption clearly participates in regulating nutrient and contaminant transport in soil. Chemical and physical forces act during adsorption in direct relation to soil-surface functional groups and the ion or molecule of the solution. The interplay between both these relations gives rise to surface complexes that can be classified as either internal or external sphere complexes. Internal sphere complexes are established by chemical forces that are generally irreversible and slightly affected by changes in ionic strengths. In turn, external sphere complexes primarily involve Coulombic interactions that, through a reversible process, are affected by ionic strengths in the aqueous phase [23].

Most soils are heterogeneous and constituted by different minerals, solids, and organic compounds. Various interaction mechanisms of soil with heavy metals have been described, including diffusion through micropores and adsorption at sites with variable reactivity. It is not possible to discriminate between these mechanisms, being more appropriate to use the term "sorption" in order to describe the retention of heavy metals by these three pathways [24]. The type of sorption and metal-binding mechanisms depend on various factors, such as ionic radius, electronegativity, surface type, valence electrons, and ionic strength of the solution. Currently, strict regulations exist for metals due to residual accumulations and persistence in the environment, as supported by findings after specific contamination events [25, 26]. Furthermore, a number of studies have established the threat posed by the possible contamination of water and soil resources destined for agricultural ends. Any subsequently produced plants would represent healthy risk to consumers [27–29].

3. Metals in honey

The presence of metals in honey has been associated with the presence of hives close to contamination sources, such as factories, highways, volcanoes, or mines/mine tailings. Contamination sources can also include agrochemicals that contain cadmium and arsenic, among others [30–32]. Due to this association, extensive research has been conducted in honey to determine the relationship between heavy metal contents and quality indicators or biological markers [33, 34]. Frequently, heavy metal concentrations in honey are low, complicating the analysis of these elements. This complication is directly evidenced in the quality of obtained results, where any loss during the analytical processing of samples influences the concentration values determined for each metal [35].

Related to the analysis of honey, Przybyłowski and Wilczyńska [36] conducted research on polyfloral honey produced in Poland to evaluate possible relationships between parameters such as pH, the glucose:fructose ratio, moisture, electric conductivity, and hydroxymethylfurfural concentration, among others, and the presence of cadmium, lead, and zinc. These relationships were determined based on methodologies established by the Association of Analytical Communities [37] for processing organic samples and performing posterior metal assessments. While no clear relationships were found between the measured parameters and the metals studied, discrete cadmium and lead concentrations were found in all of the studied samples. This finding indicates a degree of environmental contamination. Similarly, relationships did exist between plant origin and the presence of zinc in samples. Further research was conducted by Hernández et al. [38], who analyzed the metal contents in 81 honey samples from the Canary Islands and compared results against 35 additional samples from zones in Spain and Europe in general. Analyses established that the concentrations of alkaline and alkaline earth metals were within specific ranges that discriminated between Canary Islands and European mainland honey. The authors therefore concluded that this type of analysis can be used to certify the source of a honey. Hernández et al. [38] also suggested that the presence of metals could indicate the production of honey in areas contaminated by these metals.

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Another study on avocado honey from Spain also showed a common pattern between the measured concentration of alkaline and alkaline earth metals. Just as with the Cannery Island honey, Terrab et al. [39] suggested that honey origin could be established based on the concentrations of certain metals. However, the correlation between botanic origin and the presence of metals has been difficult to establish in other parts of the world. For example, Fredes and Montenegro [40] studied the possible origin-metals correlation for honey from distinct regions of Chile, but were unable to establish an association between the presence of the measured elements and the botanic/geographic origin of the analyzed honey samples.

On the other hand, an objective established by a number of researchers has been to correlate the presence of metals with the biological properties of honey. One such investigation by Küçük et al. [41] analyzed three honey samples with different botanic sources in Turkey to evaluate a possible relationship between the concentrations of alkaline, alkaline earth, and other metals with the antibacterial properties of honey. The obtained results were able to establish that honey with higher concentrations of all the studied metals also presented greater antibacterial activities. In the three samples, the metal concentrations did not exceed permitted limits. Nevertheless, no clear link was found between the measured metal concentrations and other biological properties of honey, such as phenolic compound levels.

4. Sample treatments

Before assessing the metal contents in honey, samples need to be pretreated to eliminate the majority of organic matrix components that can interfere in obtaining results. One method used in determining metal contents is solid phase extraction. This method can remove the predominant sugars from honey, thereby allowing for the collection of concentrated metal extracts that can then be analyzed through atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectrometry (ICP-OES), or inductively coupled plasma mass spectrometry (ICP-MS).

Solid phase extraction can be useful in fractioning extracts of an element, zinc for example, that could be present in honey as hydrophobic complexes or as cationic species. Resins, such as Amberlite XAD-16 and Dowex-x8-200, must be used in these cases to accurately separate metal species [42]. Other strong cation-exchange styrene-divinylbenzene resins, including Amberlite IRP-69, Dowex 50W x8-400, and Dowex HCR-W2, have been used to determine and fractionate manganese and zinc contents in extracts [43]. Similarly, Dowex 50W x8-400 and Dowex HCR-W2, together with the Diaion WT01S resin, have been used to satisfactorily detect copper and zinc species [44]. Solid phase extraction is advantageous because it destroys all of the organic materials present in honey samples, thereby reducing analysis time and risks of analyte loss that could affect result reliability. However, application of this method is limited when a mix of various metals is needed for subsequent analyses.

Recently, a new chelating resin of poly[2-(4-methoxyphenylamino)-2-oxoethyl methacrylate-co-divinylbenzene-co-2-acrylamido-2-methyl-1-propanesulfonic acid] was synthesized for determining Cd(II), Co(II), Cr(III), Cu(II), Fe(III), Mn(II), Pb(II), and Zn(II) ions. This resin

showed good performance in separation and preconcentration of those trace metals with acceptable recovery values (higher than 95%) in comparison with other reported methods [45].

Another methodology with a purpose similar to solid phase extraction is wet digestion, which applies strong acids to digest organic material in honey. Specifically, samples must be heated for 3–4 h at 105°C to remove as much water as possible. Following this, digestion takes place at 45°C through the addition of an aliquot composed of an acid mix (i.e., HNO_3/HCl 1:1) until the organic matter is fully destroyed. Excess acid is then evaporated through drying. Finally, the obtained ashes are suspended in 10 mL HNO_3 10% v/v. The resulting solutions can be directly measured via AAS, ICP-OES, or ICP-MS [46]. On variation of wet digestion is calcination in a muffle furnace, which produces ashes that can then be suspended in a solution of 0.1 M HNO_3 and H_2O_2 at 3–30% v/v [47, 48]. A noted advantage of this method is that it permits measurement of diverse analytes through only one approach. However, a disadvantage is the risk for cross-contamination between samples and the time of analysis, so close supervision is needed during the execution of experimental procedures. Another variation on wet digestion that has been implemented with notable success is that of using microwaves to induce wet digestion [49].

Tuzen et al. [50] evaluated the efficiency of calcination with a muffle furnace as compared to other ash-generating techniques, such as wet digestion using inorganic acids and through microwave. For this, various honey samples were assessed and submitted to three digestion procedures. The obtained results for copper, magnesium, zinc, iron, lead, cadmium, and nickel, among others, were classified according to the standard deviation obtained for each measurement. From the resulting values, the authors concluded that microwave digestion gave the best results, followed by direct wet digestion. Finally, calcination via a muffle furnace resulted in the least precise and most disperse results.

Currently, no technique has been validated for determining and measuring metals specifically in honey. The AOAC [37] lists calcination in a muffle furnace as the official method for determining metals in any organic sample. However, the application of this technique to honey is limited due to the chemical properties of distinct metals and the different ranges in which each type of metal can exist in a honey sample. The behavior of any sample during calcination is fundamentally determined by the organic composition of the sample. Preventing losses in the interior of the muffle furnace is a complicated process to control, directly affecting the distribution of the data obtained from muffle furnace measurements. Furthermore, although metals are often collectively referred to as a single group of elements, metals present important physico-chemical differences. These variations constitute another challenge during calcination via a muffle furnace. Specifically, the chances of cross-contamination within the muffle furnace are high, ultimately influencing the distribution of the obtained values.

Likewise, the toxicity to human health presented by metals varies from one element to the next. Some heavy metals, such as lead, mercury, and cadmium, are highly toxic and are found at much lower concentrations than other elements. Although there are not maximum residue levels for these elements, the World Health Organization and Food and Agriculture Organization have established acceptable levels for honey (i.e., Pb: 25 μg/kg ; Hg: 5 μg/kg; and Cd: 7 μg/kg; [51]). Therefore, sample loss during the process of obtaining ash can result in imperceptible differences between the actual and recorded values for the aforementioned

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elements. This is a relevant issue when considering the low maximum residue levels permitted, where any loss can cause statistically significant differences between classifying a honey as contaminated or uncontaminated by these elements.

5. Analytical methods

In recent years, much investigation has been focused on developing new methods for measuring metal concentrations in honey, with the aims of obtaining more reliable and exact values. Electrochemical techniques are one such option and have already shown more sensitive detection limits for some elements. One of these techniques is that samples are subjected to combined acid mineralization and microwave calcination before posterior analyses, with results evidencing good reproducibility for the quantification of copper, lead, cadmium, and zinc concentrations in eucalyptus honey [52]. Similarly, Buldini et al. [53] measured metal concentrations in various types of honey using hydrogen peroxide-mediated digestion and posterior analyte quantification using ionic chromatography or voltamperometry. The results from this method were satisfactory when compared against values obtained for the same samples by traditional methods. Nevertheless, the proposed method was determined only reliable for investigative ends as the large volumes of hydrogen peroxide needed to process each sample translate into a notable risk that would be difficult to implement and manage on an industrial scale. Moreover, higher sample quantities would also be required.

A distinct strategy for the analysis of metals through electrochemical techniques was proposed by Muñoz and Palmero [54]. Specifically, honey samples were diluted in hydrochloric acid, a solution to which gallium nitrate was then added to decrease any interferences that could complicate adequate zinc measurements. This method provided better results not only for zinc, but also for cadmium and lead in the assessed honey. The primary advantage of the technique proposed by Muñoz and Palmero [54] is that digestion through H_2O_2 was not used. Nevertheless, this technique was unable to measure elements such as copper, thus limiting its widespread application.

In general, metals are quantified through traditional methods such as AAS, ICP-OES, and ICP-MS due to high instrument sensitivities. While one-third of all honey mineral contents is potassium (K), elements frequently found in trace amounts include iron, copper, and manganese, among others [55, 56]. For the more predominant inorganic elements in honey, AAS is the most convenient measurement method [43, 57]. However, when the elements under study exist in lower concentrations, then the use of more highly sensitive techniques should be preferred, using ICP-MS as the primary option and ICP-OES as the secondary option [51, 58, 59]. In many cases, ICP-MS has been used for determining metals in other related products obtained from bees. The analysis of metal contents in honeybee venom showed that this equipment permits achieving very low levels for quantifying of As, Ba, Pb Cd, Sb and Cu. This tool is important when honeybee venom is a recommended treatment for certain diseases in medicine [60]. Whatever the analysis of honey, pollen, or any other product taken from beehives, it is important to note that the ICP-MS requires several steps to be considered before chemical analysis. In order to achieve reliable results, it is advisable the optimization of the

instrument including calibration with standard solutions, fortification of samples, and the use of a reference material. Also, for having a correct validating process for one analytical method, it is necessary to incorporate a confirmation method to obtain quality data. These last analyses may be performed using graphite furnace atomic absorption spectrometry [61].

6. Conclusions

Several regions in the world are suitable for honey production with different attributes due to the presence of melliferous species. However, in many cases, the apicultural activities, beehives, and the melliferous plants occur near of sources of pollution and it may produce certain changes in the composition of honey.

In the last years, the foreign trade has increased the demand for honey and beehive products without toxic residues for fulfillment of food safety policies.

Since honeybees are able to fly even 4 km per day as a maximum distance from the beehive to the floral source for collecting nectar, it is possible to detect certain undesirable compounds and/ or residual molecules from different human activities. These pollutants can be deposited onto the surfaces of melliferous plants and flowers, especially in the case of plants growing near industries, highways, or volcanoes. Likewise, abiotic factors such as air, water, and soils may be polluted with metals and they may play an important role in transferring residues to honey.

Metals are listed among the pollutant residues that can be detected in the final composition of honey, and besides, they are classified as the main group of inorganic pollutants. They can be toxic for human beings if found at levels above the permitted limits, due to damages to physiological functions of living systems and their persistence through the food chains.

One of the most remarkable problems in metals analysis is related to very low concentrations available in honey content. In addition, honey is a complex organic matrix and it has to be treated previously to chemical determination of metals by classic analytical methods for instance, inductively coupled plasma (ICP-OES) or atomic absorption spectroscopy (AAS). The chemical treatment of samples related to extraction methodologies of metals includes the calcination process and/or acid digestion. These last steps are mainly responsible for the quality of the obtained products because any loss of mass will be reflected in no reliable values.

In that way, it is very important to determine the presence of heavy metals in honey using analytical procedures to obtain reliable values. It must be considered that honey and/or another beehive product such as bee pollen, propolis, or beeswax are organic matrix and thus, a cleanup method of samples before chemical analysis for determining heavy metal content is essential for achieving optimal results.

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Chapter 15

Residue Determination in Honey

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Additional information is available at the end of the chapter

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Abstract

The use of antibiotics to fight bacterial and fungal honeybee diseases is documented since 1940s. Although at present in some countries certain antibiotics are authorized in apiculture, only few law systems provide maximum residue limits in honey. In addition, residues of worldwide banned antibiotics such as chloramphenicol, nitrofurans and nitroimidazoles have been frequently found. Therefore, the availability of reliable analytical methods able to detect concentrations at few parts per billions is fundamental. After a general overview of the available sample treatment strategies and analytical techniques, the most significantly published methods are discussed. Aminoglycosides and, to a lesser extent, tetracyclines are the more difficult classes to analyse. The current trends are the development of multiclass procedures and of micro-extraction techniques to improve the cost-effectiveness of residues control in the globalization era.

Keywords: honey, antibiotics, honeybee diseases, sample preparation, liquid chromatography-mass spectrometry

1. Introduction

Antimicrobials are used in food-producing animals to prevent and/or treat animal diseases. Although epidemiological data on the real magnitude of their adverse effects are very scarce, they indicate that the presence of antibiotic residues in food could be an important vehicle for the development of antibiotic-resistant bacterial strains. Because of these concerns, many countries have restricted the use of antibiotics in farm. The major honeybee diseases for which antibiotics are applied are American foulbrood, European foulbrood infections and nosemosis. Foulbrood infections are caused by bacteria, whereas *Nosema* disease is caused by a fungus. Currently, in the European Union the maximum residue limits (MRLs) for antibiotics in food are listed in Regulation (EU) No 37/2010 [1]. This regulation stipulates that each antibiotic

must have a MRL before it can be used on a food-producing animal. European Union does not allow the use of antibiotics for treatment of honeybees, and therefore, there are not MRLs in honey for these substances. The lack of harmonized rules with regard to acceptable control methods, limits of detection or sampling methods, results in different interpretations by European Member States. Some Member States and Switzerland have established action limits or tolerance levels [2, 3]. In the CRL Guidance Paper (2007) [4], the European Union References Laboratories (EURLs, ex CRLs) proposed recommended concentrations for analysis of macrolides, streptomycin, sulphonamides and tetracyclines in honey within the national residue control plans carried out in accordance with Council Directive 96/23/EC [5] (**Table 1**). These recommended concentrations, however, have no real legal basis. They are used as reference during method development since detection capability (CCβ) for screening methods or decision limit (CCα) for the confirmatory ones [6] should be lower than recommended concentrations. All the veterinary drugs just mentioned belong to the Group B of Annex I of Council Directive 96/23/EC [5], that is, they are permitted substances with fixed MRLs in several food commodities. On the other hand, in the case of banned substances (Group A, Annex I of Council Directive 96/23/EC) such as chloramphenicol (CAP) and nitrofurans (NFs), the European Union has set minimum required performance limits (MRPLs) of 0.3 and 1.0 μg/kg, respectively. MRPLs are foreseen in Article 4 of Commission Decision 2002/657/EC

Country	Approved substance	MRLs[a] (μg/kg)	RCs[b] (μg/kg)	Source
EU	Streptomycin	–	40	European Regulation 37/2010 [1] CRL Guidance Paper [4]
	Tetracyclines	–	20	
	Sulphonamides	–	50	
	Erythromycin Tylosin	–	20	
USA	Lincomycin	–	–	CFR—Code of Federal Regulations—Title 21 [8]
	Oxytetracycline	–	–	
	Tylosin	–	–	
Canada	Fumagillin	25	–	List of Maximum Residue Limits (MRLs) for Veterinary Drugs in Foods [9]
	Oxytetracycline	300	–	
	Erythromycin	–	30[c]	
	Tylosin	200[d]	–	
Australia/New Zealand	Oxytetracycline	300	–	Food Standards Code (standard 1.4.2—Schedule 20) [10]
Japan	Oxytetracycline	300	–	Positive List System for Agricultural Chemical Residues in Foods [11]
	Amoxicillin	8	–	
	Ampicillin	9	–	

[a]Maximum residue limits (MRLs) or tolerances (legal limits).

[b]Recommended concentrations (RCs) which only represent a reference point for analytical method performances.

[c]Working residue level (WRL) below which there is considered to be undue risk to human health.

[d]Sum of tylosin A and B.

Table 1. Worldwide limits for antibiotics in honey.

[6] and they are reference point for action. They are intended to harmonize the analytical performance of methods ensuring the same level of consumer protection in the European Union. Among banned substances, also the use of nitroimidazoles has been documented in beekeeping practice. However, no MRPLs have been fixed for nitroimidazoles, the European Union has not fixed the relevant MRPLs, and during the development of analytical methods, the recommended concentration of 3 μg/kg (CRL Guidance Paper [4]) is taken into account.

The worldwide standard, the Codex Alimentarius, has not fixed any MRL for antibiotics in honey [7]. The Codex Alimentarius or "Food Code" was established by FAO and the World Health Organization in 1963 to develop harmonized international food standards, which protect consumer health and promote fair practices in food trade. Similarly, in United States, no tolerances for antibiotics in honey have been established, although oxytetracycline is approved for longtime in beekeeping practice to control American foulbrood. At present, lincomycin and tylosin are authorized, too. The MRLs (or tolerances) for residues of antibiotics in food are set by the US Food and Drug Administration (USFDA) and listed in the Code of Federal Regulations, Title 21 [8]. Conversely, Canada, Australia, New Zealand and Japan have established MRLs for oxytetracycline (300 μg/kg) [9–11]. In addition, also MRLs for fumagillin, oxytetracycline and tylosin are provided by Canadian authorities (**Table 1**). For erythromycin, a working residue level is provided, below which no risk to human health is considered.

Sulphathiazole and oxytetracycline are probably the first antibiotics used to fight honeybee diseases. Starting from 1980s, analytical methods have been developed for these two drugs in honey at trace levels mainly based on liquid chromatography coupled to UV-Vis (LC-UV-Vis) and fluorescence detectors (LC-FLD). In the early 2000s, the availability of liquid chromatography systems coupled to mass spectrometric analysers (LC-MS) at bench level involved the progressive development of procedures using this technique which allows a more selective and universal detection than the traditional detectors based on UV absorption (quite universal, but not selective) or fluorescence (selective, but not universal). Therefore, existing methods have been progressively converted using LC-MS improving performances and sample throughput, and new challenging analytical problems have been solved thanks to this technique equipments (e.g. the detection of nitrofurans metabolites in food).

From a toxicological point of view, in the European Union law system the distinction is between permitted drugs (aminoglycosides, lincomycin, macrolides, quinolones, sulphonamides and tetracyclines) and banned drugs (chloramphenicol, nitrofurans and nitroimidazoles) belonging to substances of group B and A, respectively (Annex I of Directive 96/23 [5]). As discussed before, there are not MRLs for antibiotics in honey (**Table 1**). Hence, in this context, "permitted drugs" are drugs with an MRL in food commodities other than honey (meat, milk, liver, etc), whereas the banned ones (chloramphenicol, nitrofurans and nitroimidazoles) cannot be used in any food-producing species generally not only in European Union, but also in several other countries. This distinction is also fundamental to choose the analytical technique to develop confirmatory methods, which are procedures fulfilling Commission Decision 2002/657/EC criteria [6]. For the banned substances, the use of mass spectrometric detectors is mandatory, whereas, for the permitted ones, traditional detectors, UV-Vis or FLD, are suitable, too. In addition, for banned drugs, the required method limits are in the range from 0.1 to 1 μg/kg; for permitted drugs, limits of about one order of magnitude greater can be acceptable (**Table 1**).

The use of liquid chromatography coupled to mass spectrometry equipments and the worldwide improvement of law systems probably explains the decrease in the incidence of veterinary drug residues in honey and honeybee products (royal jelly and propolis). The number of cases per year in 2003 was 40, whereas in 2015 only six notifications have been recorded by the European Rapid Alert System for Food and Feed (RASSF) as shown in **Table 2** [12]. In place since 1979, RASSF enables information to be shared efficiently between its members [national food safety authorities of EU Member States, the EU Commission, the European Food Safety Authority (EFSA), Norway, Liechtenstein, Iceland and Switzerland]. It provides an efficient service to ensure that urgent notifications are sent, received and responded to in the shortest time possible. Thanks to RASSF, many food safety risks had been averted before any harm to European consumers was caused.

Year	No of notifications	No of found substances[a]	Number (substance)
2002	45	57	34 (CAP), 13 (STR/DSTR), 7 (SAs), 3 (TCs)
2003	40	53	20 (SAs), 17 (CAP), 11 (STR), 3 (TCs), 2 (NFs)
2004	25	27	10 (SAs), 7 (CAP), 5 (NFs), 5 (STR)
2005	41	49	25 (CAP), 8 (STR), 6 (SAs), 5 (TCs), 4 (NFs), 1 (MAC)
2006	16	17	7 (CAP), 6 (SAs), 2 (STR), 1 (NFs), 1 (TCs)
2007	20	41	24 (SAs), 6 (QNs), 6 (TCs), 2 (MACs), 1 (CAP), 1 (STR), 1 (NFs)
2008	27	29	9 (MACs), 7 (TCs), 5 (SAs), 3 (CAP), 2 (QNs), 2 (STR), 1 (NFs)
2009	10	10	4 (TCs), 3 (NFs), 2 (STR), 1 (SA)
2010	8	9	3 (lincomycin), 2 (STR), 1 (TC), 1 (NMZ), 1 (MAC), 1 (QN),
2011	6	6	3 (SAs), 2 (NMZs), 1 (lincomycin)
2012	6	7	5 (SAs), 2 (NFs),
2013	4	4	2 (SAs), 1 (NFs), 1 (TCs),
2014	1	1	1 (SA—sulphamethoxazole)
2015	6	10	3 (CAP), 3 (STR/DSTR), 2 (TCs), 1 (NMZ), 1 (SA)

[a]In the same sample more than one residue could be present.

Table 2. RASFF notifications in the period 2002–1015 (hazard category "residues of veterinary medicinal products"; product category "honey and royal jelly").

In Section 4, an extensive overview of the main published analytical methods for the determination of residues of the antibiotics (legally or illegally) used in apiculture is carried out. The analytical steps of each selected method (sample treatment, analytical technique and detection limits) are summarized in **Tables 3–11**.

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
CAP	100 mM NaAc buffer (pH 5.0)/ Oasis HLB-SPE, phosphate buffer (pH 6.5), ACN:DCM (80:20, v/v)	Symmetry Shield RP18 (150 × 2.1 mm, 3.5 μm)	Gradient: water/ ACN	LC-MS/MS (ESI−)	0.021	[17]
CAP	50 mM NaAc buffer (pH 5.2)[b]/ DCM (Extrelut[c])	Acquity UHPLC BEH C18 (50 × 2.1 mm, 5.0 μm)	Gradient: 25% NH_3 in 10% CAN/25% NH_3 in ACN	LC-MS/MS (ESI−)	0.013	[18]
CAP	Water/ACN, $CHCl_3$	Purospher Star RP-18 (55 × 4.0 mm, 3.0 μm)	Gradient: 0.15% FA/MeOH	LC-MS/MS (ESI−)	0.01	[19]
CAP	Water/MIP-SPE	Ascentis C18 (100 × 2.1 mm, 3.0 μm)	Isocratic: 30% ACN in 10 mM NH_4Ac (pH 6.7)	LC-MS/MS (ESI−)	0.03	[20]
CAP	Water/ MWCN-SPE	Halo fused-core C18 (50 × 2.1 mm, 2.7 μm)	Gradient: 0.1% FA/ACN	LC-MS/MS (ESI−)	0.008	[21]
CAP	Water/EtAc	Luna C18 100 Å (50 × 2.0 mm, 5.0 μm)	Gradient: 2 mM NH_4Ac/MeOH	LC-MS/MS (ESI−)	0.10	[22]
CAP and FF, FFA, TAP	1% NH_3/Oasis HLB-SPE	Ascentis express phenyl-hexyl (100 × 2.1 mm, 2.7 μm)	Gradient: 5 mM NH_4Ac buffer (pH 5.0)/MeOH	UHPLC-MS/ MS (ESI−)	0.03	[23]
CAP	Water/EtAC	Luna C18 (150 × 4.6 mm, 5.0 μm)	Gradient: Water/ ACN	LC-MS/MS (ESI−)	0.09	[24]
CAP and FF, TAP	QuEChERS (1% AcOH in ACN)	LMA-MAA-EDMA monolithic[d] (150 mm × 250 μm)	Gradient: Water/ ACN-MeOH (90:10, v/v)	LC-MS/MS (ESI−)	0.045	[25]

[a]FF, florfenicol; FFA, florfenicol amine; TAP, thiamphenicol.

[b] An enzymatic hydrolysis was carried out to deconjugate CAF in the muscle sample included in the method scope.

[c]Extrelut (diatomaceous earth) was used to help the liquid-liquid extraction process.

[d]LMA-MAA-EDMA: poly(lauryl methacrylate-co-methacrylic acid-co-ethylene glycol dimethacrylate).

Table 3. Confirmatory methods for chloramphenicol (CAP).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
LIN, TYL	Na_2CO_3–$NaHCO_3$ buffer (pH 9.0)/ C18-SPE	Zorbax C8 (150 × 2.1 mm, 5.0 μm)	Gradient: 0.1% TFA/0.1% TFA in ACN/MeOH	LC-MS (APCI+)	7–10	[31]
ERY, OLE, SPI, TILM, TYL	100 mM NaH_2PO_4 buffer at pH 8.0/Oasis HLB-SPE	YMC ODS-AQ S-3 120 Å 50 × 2.0 mm	Gradient: 1% FA/water/ACN	(a) LC-MS (ESI+) (b) LC-MS/MS (ESI+)	(a) <1 (b) 0.01–0.07	[32]
ERY, LIN, JOS, SPI, TILM, TYL	TRIS[b] buffer (pH 10.5)/Oasis HLB-SPE	Synergi Hydro-RP (150 × 2.0 mm, 4.0 μm)	Gradient: 10 mM NH_4Ac (pH 3.5)/ACN	LC-MS/MS (ESI+)	0.24–2.10	[33]
TYL, TYLB, TYLC, TYLD	100 mM Na_2CO_3–$NaHCO_3$ buffer (pH 9.0)/ Strata-X-SPE	Luna C18(2) 100 Å (150 × 4.6 mm, 5.0 μm)	Gradient: 1% FA/ACN/ MeOH	(a) LC-MS (ESI+) (b) LC-DAD	(a) 2–3 (b) 49–57	[34]
ERY, NEO, OLE, SPI, TILM, TYL, TYLB	100 mM NaH_2PO_4 buffer (pH 8.0)/ Oasis HLB-SPE	(a): YMC ODS-AQ S-3 120 Å (50 × 2.0 mm) (b): Acquity BEH C18 (100 × 2.1 mm, 1.7 μm)	(a) Gradient:10 mM NH_4Ac/ ACN (b) Gradient: Water/1% FA/ ACN	(a) LC-MS/MS (ESI+) (b) UHPLC-HRMS/MS (Q-TOF) (ESI+)	(a) 0.01–0.5 (b) 0.2–1.0	[35]
ERY, TYL	100 mM Na_3PO_4 (pH 8.0)/C18-SPE	Gemini C18 110 Å (50 × 2.0 mm, 5.0 μm)	Isocratic: water/ACN (30:70, v/v)	LC-MS/MS (ESI+)	5.0–5.2	[36]
AIVT, AZI, CLA, ERY, JOS, SPI, TILM, TYL	Water/Oasis HLB-SPE	C18HCE (100 × 2.1 mm, 5.0 μm) (home-made)	Gradient: 0.2% FA/0.2% FA in ACN	LC-MS/MS (ESI+)	0.01–0.5	[37]

[a]AIVT, acetylisovaleryltylosin (tylvalosin); AZI, azithromycin; CLA, clarithromycin; ERY, erythromycin; JOS, josamycin; LIN, lincomycin; NEO, neospiramycin; OLE, oleandomycin; SPI, spiramycin I; TILM, tilmicosin; TYL, tylosin A; TYLB, tylosin B; TYLC, tylosin C; TYLD, tylosin D.
[b]TRIS, tris(hydroxymethyl)aminomethane.

Table 4. Confirmatory methods for lincomycin and macrolides (MACs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
AHD, AMOZ, AOZ, SEM	[b]/EtAc, Lichrolut EN-SPE	Symmetry Shield C18 (150 × 2.1 mm, 3.5 μm)	Gradient: 0.025% AcOH/ ACN	LC-MS/MS (ESI+)	0.12–0.56	[39]
AHD, AMOZ, AOZ, SEM, Ft, Fz, Nt, Nz	100 mM HCl/ Oasis HLB-SPE[c], AF buffer to pH 6–7, Oasis HLB-SPE	Inertsil ODS3 (150 × 2.0 mm, 3.0 μm)	Gradient: 20 mM AF buffer (pH 3.8)/ACN	LC-MS/MS (ESI+)	0.15–2.1	[40]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
AHD, AMOZ, AOZ, SEM	10% NaCl/Oasis HLB-SPE[c], (NaCl), hexane, EtAc	Inertsil ODS3 (150 × 2.1 mm, 3.5 μm)	Gradient: 10 mM NH_4Ac/ MeOH	LC-MS/MS (ESI+)	0.2	[41]
AHD, AMOZ, AOZ, SEM	[b]/hexane, EtAc	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	Gradient: 0.5 mM NH_4Ac/ MeOH	UHPLC-MS/MS (ESI+)	0.09–0.14 (CCα)	[42]
AHD, AMG, AMOZ, AOZ, DNSH, NPIR, PSH, SEM and CAP	[b]/EtAc, Oasis HLB-SPE	Acquity UHPLC BEH C18 (50 × 2.1 mm, 1.7 μm)	Gradient: 0.37% NH_3 in 10 mM NH_4Ac:MeOH (80:20, v/v)/ MeOH	UHPLC-HRMS/ MS (Q Exactive Plus) (ESI±)	0.05–2.3	[43]
AHD, AMOZ, AOZ, SC	[b]/EtAc, hexane	Synergy Hydro-RP (150 × 2.0 mm, 4.0 μm)	Gradient: water:MeOH (80:20, v/v)/0.1% AcOH in MeOH	LC-MS/MS (ESI+)	0.22–0.57	[44]
AHD, AMOZ, AOZ, SEM and DMZ, RNZ	[b]/QuEChERS (ACN) without d-SPE	Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5.0 μm)	Gradient: 5 mM AF buffer in water:MeOH (90:10, v/v) (pH 3.0)/MeOH	LC-MS/MS (ESI+)	0.21–0.53	[45]

[a]AHD, nitrofurantoin metabolite; AMG, nitrovin metabolite; AMOZ, furaltadone metabolite; AOZ, furazolidone metabolite; DMZ, dimetridazole; DNSH, nifursol metabolite; Ft, furaltadone; Fz, furazolidone; Nt, nitrofurantoin; Nz, nitrofurazone; NPIR, nifurpirinol; PSH, nifuroxazid metabolite; RNZ, ronidazole; SEM, nitrofurazone metabolite.

[b]Derivatization with 2-NBA in HCl solution with subsequent neutralization.
[c]Derivatization with 2-NBA and HCl after the indicated purification step.

Table 5. Confirmatory methods for nitrofurans (NFs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
DMZ, HMNNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ, TNZ, TRZ[b] and CAP	Water/ACN (NaCl), hexane	Zorbax Eclipse Plus C18 (100 × 2.0 mm, 1.8 μm)	Gradient: 0.1% AcOH/0.1% AcOH in ACN	LC-MS/MS (ESI ±)	0.13–2.0	[47]
DMZ, MNZ, RNZ	Water ($NaHCO_3$)/ EtAc, Silica-SPE	Sunniest C18 (150 × 2.0 mm, 3.0 μm)	Gradient: water/MeOH	LC-MS/MS (ESI+)	0.05–0.2	[48]
DMZ, HMNNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ	Water/QuEChERS (without d-SPE), Alumina-N-SPE	Pentafluorophenyl-propyl-bonded silica (150 × 2.0 mm, 3.0 μm)	Gradient: 0.01% AcOH/ ACN	LC-MS/MS (ESI+)	0.03–0.15	[49]
DMZ, HMNNI, MNZ, MNZ-OH, RNZ	0.1% FA, 10 mM NH_3 to pH 7.0/ Strata-SDB[c]-SPE	Kinetex XB C18 (100 × 3.0 mm, 2.6 μm)	Isocratic: 0.1% FA/MeOH (88:12, v/v)	LC-MS/MS (ESI+)	0.05–0.1	[50]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
DMZ, HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ, SCZ, TRZ[b]	10 mM NH_4Ac (pH 6.0)/MIP	Kinetex XB C18 (150 × 2.1 mm, 2.6 μm)	Gradient: 0.1% FA/0.1% FA in ACN	LC-MS/MS (ESI+)	0.18–0.51	[51]

[a]DMZ, dimetridazole; HMMNI, 2-hydroxymethyl-1-methyl-5-nitroimidazole; IPZ, ipronidazole; IPZ-OH, ipronidazole metabolite; MNZ, metronidazole; MNZ-OH, metronidazole metabolite; RNZ, ronidazole; TRZ, ternidazole; CAP, chloramphenicol.

[b]Other less common NMZs are included in the method scope.
[c]Styrene-divynilbenzene copolymer (RP-SPE).

Table 6. Confirmatory methods for nitroimidazoles (NMZs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
(a) CIPRO, DANO, ENRO, MARBO, NOR, SARA[b] (b) FLUME, NALI, OXO	(a) 2% AcOH in ACN, SCX-SPE (b) ACN, 50 mM Na_2HPO_4 pH 11.0, SAX-SPE[c]	(a) Zorbax RX C8 (250 × 4.6 mm, 5.0 μm) (b) Kromasil C8 (250 × 3.2 mm, 5.0 μm)	(a) Isocratic: 10 mM Phosphate buffer (pH 3.0)/ ACN (b) Isocratic: 10 mM OA/ ACN/MeOH (60:30:10, v/v/v)	LC-FLD	5–50	[52]
CIPRO, DANO, DIFLO, ENRO, FLUME, MARBO, NALI, NOR, OXO, SARA	NaH_2PO_4/ Na_2HPO_4 buffer (pH 6.3)/ hexane, Oasis HLB-SPE	XBridge MS C18 (100 × 2.1 mm, 3.5 μm)	Gradient: 1% FA/ACN	LC-MS/MS (ESI+)	0.07–0.66	[53]
CIP, DAN, DIFLO, ENRO, NOR, SARA[b]	MacIlvaine buffer (pH 4.0) (Na_2EDTA)/ Oasis HLB-SPE, MCAC-SPE	Inertsil ODS-4 (150 × 4.6 mm, 3.0 μm)	Isocratic: 1 mM SDS, 20 mM citrate buffer (pH 3.1)/ACN (70:30, *v/v*)	LC-FLD	0.33–4.4	[54]
CIPRO, DANO, DIFLO, ENRO, MARBO, SARA[b]	ACN/hexane	WondaSil C18 (150 × 4.6 mm, 5.0 μm)	Isocratic: 1% FA/MeOH (71:29, v/v)	LC-DAD	0.4–19	[55]
CIPRO, DANO, DIFLO, ENRO, FLUME, MARBO, OXO	30 mM NaH_2PO_4 buffer (pH 7.0)/ QuEChERS (5% FA in ACN)	Zorbax Eclipse Plus HHRD (50 × 2.1 mm, 1.8 μm)	Gradient: 0.02% FA/ACN	UHPLC-MS/ MS (ESI+)	0.2–1.7	[56]
CIP, ENR, NOR	Water, H_2SO_4 to pH 1.0/ PS-MSLM[d]	Zorbax Eclipse XDB-C18 (150 × 4.6 mm, 5.0 μm)	Isocratic: MeOH/ ACN/0.34% PA, 0.6% TEA[e] (15:5:80, v/v)	LC-FLD	0.067–0.35	[57]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
ENRO, FLUME, MARBO, OXO	50 mM SDS (pH 3.0)	Kromasil C18 (150 × 4.6 mm, 5.0 μm)	Isocratic: 50 mM SDS, 10 mM NaH_2PO_4, HCl to pH 3.0/TEA[e]/1-propanol (87:12.5:0.5, v/v/v)	LC-FLD	10–100	[58]

[a]CIPRO, ciprofloxacin; DANO, danofloxacin; DIFLO, difloxacin; ENRO, enrofloxacin; FLUME, flumequine; MARBO, marbofloxacin; NALI, nalidixic acid; NOR, norfloxacin; OXO, oxolinic acid; SARA, sarafloxacin.

[b]Other less common QNs are included in the method scope.

[c]SCX: strong cation exchange and SAX: strong anion exchange.

[d]PS-MSLM: phase separation-based magnetic-stirring salt-induced liquid-liquid microextraction method (LLE).

[e]Triethylamine.

Table 7. Confirmatory methods for quinolones (QNs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
STR	10 mM $HClO_4$ (pH 2.0)/SCX-SPE, C18-SPE	Hypersil BDS (100 × 4.0 mm, 3.0 μm)	Isocratic: 10 mM AHS[b], 0.4 mM, NQS in 20% ACN (pH 3.3)/ACN (97:3, v/v)	LC-FLD (post-column derivatization)	5	[59]
STR	Water/ WCX-SPE	Zorbax C18 (150 × 2.1 mm, 5.0 μm)	Isocratic: 20 mM HFBA[c]/ ACN (70:30, v/v)	LC-MS/MS (ESI+)	2	[60]
STR	0.1% PA/SCX-SPE, C18-SPE	Hypersil ODS2 (150 × 4.6 mm, 5.0 μm)	Isocratic: 10 mM AHS[b], 0.4 mM NQS (pH 3.3)/ACN (28:72, v/v)	LC-FLD (post-column derivatization)	5	[61]
STR, DSTR	50 mM AHS[b], [25] mM Na_3PO_4 buffer to pH 2.0/ C18-SPE	Alltima C18 (150 × 2.1 mm, 5.0 μm)	Isocratic: 1.9 mM PFPA[d], [3].2 mM AF/ ACN (85:15, v/v)	LC-MS/MS (ESI+)	<1–2	[62]
APR, AMI, DSTR, GEN, KAN, NEO, PAR, SPC, STP	Water/ WCX-SPE	ZIC®-HILIC, 150 × 2.1 mm, 3.5 μm, SeQuant AB	Gradient: 175 mM NH_4Ac (pH 4.5)/0.2% FA in ACN	LC-MS/MS (ESI+)	17–99	[63]
STR, DSTR	KH_2PO_4-Na_2EDTA-TCA buffer (pH 4.0), NaOH to pH 7.5/Oasis HLB-SPE	HILIC Atlantis (150 × 2.1 mm, 3.0 μm)	Gradient: 0.05% FA/0.05% FA in ACN	LC-MS/MS (ESI+)	19–20	[64]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
DSTR, GEN SPC, STR	20 mM K_2HPO_4 buffer (pH 7.4)/ MIP-SPE	XAmide HILIC (150 × 4.6 mm, 5.0 μm)	Isocratic: 20 mM AF (pH 3.0)/ACN (40:60, v/v)	LC-MS/MS (ESI+)	1.8–6.0	[65]
AMI, APR, DSTR, GEN, HYG, KAN, NEO, PAR, SIS, SPC, STR, TOB	Water/10 mM NH_4Ac, 0.4 mM Na_2EDTA, 0.5% NaCl, 5% TCA, 1–10 M NaOH, WCX-SPE	Obelisc R 100 Å, (100 × 2.1 mm, 5.0 μm)	Gradient: 1% FA/1% FA in ACN	LC-MS/MS (ESI+)	1–12	[66]
DSTR, GEN, SPC, STR	5 mM K_2HPO_4 buffer (pH 11.0)/PVA-Sil-SPE[f] (home-made sorbent)	TE[g]-Cys HILIC (150 × 3.0 mm, 3.0 μm)	30 mM AF/ FA (99/1, v/v)/ ACN/water/FA (80:19:1, v/v/v)	LC-MS/MS (ESI+)	2.3–6.1	[67]
APR, DSTR, GEN, NEO, PAR, SPC, STR	50 Mm KH_2PO_4 buffer (pH 7.0)/ MIP-SPE	Bare fused-silica capillary (90 cm × 50 μm × 375 μm)	200 mM FA/7 mM NH_3	CZE-IT-MS[e]	6–103	[68]

[a]AMI, amikacin; APR, apamycin; GEN, four isomers of gentamicin; HYG, hygromycin; KAN, kanamycin; NEO, neomycin; PAR, paromomycin; SIS, sisomycin; SPC, spectinomycin; TOB, tobramycin.

[b]AHS, sodium 1-heptanesulphonic acid (ion-pairing reagent).

[c]HFBA, heptafluorobutyric acid (ion-pairing reagent).

[d]PFPA, pentafluoropropionic acid (ion-pairing reagent).

[e]CZE-IT-MS: capillary zone electrophoresis coupled to ion trap mass spectrometry.

[f]PVA-Sil: polyvinyl alcohol-Silica.

[g]TE: thiol-ene.

Table 8. Confirmatory methods for streptomycin/dihydrostreptomycin (STR/DSTR).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
STZ	Acetone/1 M HCl, diethyl ether	μBondapack phenyl (300 × 3.9 mm)	Isocratic: KH_2PO4 buffer (pH 3.0) in 10% ACN	LC-UV	60	[69]
SCP, SDT, SDX, SDZ, SMP, SMR SMX, SMZ, SPD, STZ	10% TCA, 1 M Na_2HPO_4 to pH 6.5/ACN, DCM	Nucleosil C18 HD (50 × 2.0 mm, 3.0 μm)	Gradient: 0.3% FA in water:ACN (95:5, v/v)/0.3% FA in ACN	LC-MS/MS (ESI+)	<10	[70]
SCP, SDT, SMP, SMR, SMX, SPD[b]	0.1% PA (pH 2.0)/SCX-SPE, AHS[c], PA to pH 6.0, Oasis HLB-SPE	Symmetry Shield C18 (150 × 3.9 mm)	Isocratic: 10 mM KH_2PO_4 (pH 3.5–4.0)/ACN (73:27, v/v)	LC-FLD (with derivatization)	2–5	[71]

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
SCP, SDA, SDX, SGN SMP, SMR, SMT, SMZ SNL, SPD, STZ[b]	2 M HCl. 5 M NaOH and 1.2 M NaAc to pH 5.0/ACN, 1% AcOH, SCX-SPE	Purospher Star RP-18 EC (150 × 4.6 mm, 5.0 μm)	Gradient: NaAc buffer:ACN (98:2, v/v)/NaAc buffer:ACN (68:32, *v/v*)	LC-FLD (with derivatization)	1–2	[72]
SCP, SDA, SDT, SDX, SMR, SMZ, SMP, SMM, SMX, SPD, STZ[b]	0.1% PA (pH 2.0)/SCX-SPE, AHS[c], PA to pH 6.0, Oasis HLB-SPE	Atlantis dC18 (150 × 2.1 mm, 3.0 μm)	Gradient: 0.2% FA/0.2% FA in ACN	LC-MS/MS (ESI+)	0.5–6.0	[73]
SDM, SMX, STZ[b] and 4 TCs, 4 pesticides	2 M HCl/200 mM Citric acid, 20% NH_3 to pH 4.0, Oasis HLB-SPE	InertSil ODS2	Gradient: 0.2% FA/0.2% FA in ACN	LC-MS/MS (ESI+)	0.2–6.2	[74]
SCP, SDA, SDX, SMM, SMP, SMR, SMT, SMX, SMZ, SNL, SPD, SQX, STZ[b] and CAP	2 M HCl, 5% NaOH to pH 8.5/Oasis HLB-SPE	Xterra C18 (150 × 2.1 mm, 3.5 μm)	Gradient: 0.15% AcOH/0.15% AcOH in MeOH	LC-MS/MS (ESI+)	0.5–5	[75]
SDT, SDX, SMR, SMX, SMZ, SPD, STZ and DAP, TRM	2 M HCl, 300 mM Citric acid, 25% NH_3 to pH 4.0/Oasis HLB-SPE	Xterra MS C18 (150 × 2.1 mm, 3.5 μm)	Gradient: 0.1% FA/0.1% FA in ACN	LC-MS/MS (ESI+)	0.3–0.9	[76]
SDT, SDX, SDZ, SGN, SMM, SMP, SMR, SMX, SMZ, SNL, SQX, STZ[b]	300 mM Citric acid (pH 1.8)/ SCX-SPE	Symmetry C18 (100 × 2.1 mm, 3.5 μm)	Gradient: 0.2% FA/ACN	LC-MS/MS (ESI+)	2.2–17.4	[77]
SCP, SDX, SDZ, SGN, SMM, SMP, SMR, SMX, SMZ, SQX, SSZ STZ[b] and DAP, TRM	100 mM AcOH (pH 5.0)/ acetone:DCM (50/50, v/v)	Zorbax XDB-C18 (75 × 4.6 mm, 3.5 μm)	Gradient: 5 mM AF (pH 3.5)/5 mM AF in MeOH	LC-MS/MS (ESI+)	1.5–5.3	[78]

[a]DAP, dapsone; SCP, sulphachloropyridazine; SDA, sulphadiazine; SDT, sulphadimethoxine; SDX, sulphadoxine; SGN, sulphaguanidine; SMM, sulphamonomethoxine; SMR, sulphamerazine; SMP, sulphamethoxypyridazine; SMX, sulphamethoxazole; SMZ, sulphamethazine; SNL, sulphanilamide; SPD, sulphapyridine; SQX, sulphaquinoxaline; STZ, sulphathiazole; TRM, trimethoprim.

[b]And less common SAs are included in the method scope.

[c]AHS, sodium 1-heptanesulphonic acid (ion-pairing reagent).

Table 9. Confirmatory methods for sulphonamides (SAs).

Compounds[a]	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
CTC, DC, MINO, MTC, OTC, TC	MacIlvaine buffer (Na_2EDTA) (pH 4.0)/ phenyl-SPE	Discovery RP-Amide C16 (5.0 μm)	Gradient: 0.09% OA (pH 3.0)/ACN	LC-DAD	15–30	[80]
CTC, DC, OTC, TC	50 mM oxalate buffer (pH 4.0)/ Oasis HLB-SPE	Atlantis dC18 (150 × 2.1 mm, 3 μm)	Gradient: 1% FA/ ACN:MeOH (50:50, v/v)	LC-MS/MS (ESI+)	3.3	[81]
CTC, OTC, TC	MacIlvaine buffer (Na_2EDTA) (pH 4.0)/hexane, PLS-2-SPE[b], MCAC-SPE	Hydrospher C18 HS-301–3 (100 × 4.6 mm, 3.0 μm)	Isocratic: 1 M Imidazole buffer/MeOH (82:18, v/v)	LC-FLD	5–9	[82]
CTC, OTC, TC	Citrate buffer, (Na_2EDTA)/ PLS-2-SPE[b]	Tsk-gel ODS-80Ts (150 × 4.6 mm)	Isocratic: 1 M Imidazole buffer/MeOH (75:25, v/v)	LC-DAD	10–20	[83]
CTC, DC, OTC, TC	5% HCl/ MIP-SPE	Restek C18 (150 × 2.1 mm, 5.0 μm)	Isocratic: 100 mM OA/ ACN/MeOH (70:20:10, v/v/v)	LC-MS/MS (ESI+)	0.1–0.3	[84]
CTC, OTC, TC	ACN/SPE (home-made sorbent)	ShodexRSpak DE-613 (150 × 6.0 mm)	Isocratic: 0.05% TFA/ACN (60:40, v/v)	LC-MS/MS (ESI+)	3–20	[85]
CTC, OTC, TC	50 mM NH_4Ac buffer (pH 5.5)/ MCAC-SPE, Oasis HLB-SPE	Waters Phenyl (100 × 2.1 mm, 3.5 μm)	Gradient: 0.1% FA/0.1% FA in ACN:MeOH (50:50, v/v)	LC-MS/MS (ESI+)	7.2–7.7	[86]
CTC, DC, OTC, TC	MacIlvaine buffer (Na_2EDTA) (pH 4.0)/ Strata-X-SPE	Symmetry C18 (150 × 2.1 mm, 3.5 μm)	Gradient: 0.05% AcOH/0.05% AcOH in ACN	LC-MS/MS (ESI+)	5.5–9.2	[87]
CTC, DC, MTC, OTC, TC	Water/chitosan-modified graphitized MWCN	SB-C18 (50 × 4.6 mm, 5 μm)	Gradient: 0.1% FA/MeOH	LC-HRMS (Q-TOF) (ESI+)	0.61–10	[88]

[a]CTC, chlortetracycline; DC, doxycycline; DMC, demeclocycline; OTC, oxytetracycline; MINO, minocycline; MTC, methacycline; TC, tetracycline.
[b]PLS-2, polystyrene-divinylbenzene polymer (RP-SPE).

Table 10. Confirmatory methods for tetracyclines (TCs).

Compounds	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
1 QN, 16 SAs, 3 TCs (20)	2 M HCl, 300 mM Citric acid/ Oasis HLB-SPE	Nucleosil 100–5, C18 HD (50 × 2.0 mm, 5 μm)	Gradient: 0.3% FA in Water:ACN (95:5, v/v)/0.3% FA in can	LC-MS/MS (ESI+)	0.4–11	[89]
2 amphenicols, 3 AGs, 8 β-lactams, 7 MACs, 17 SAs, 5 TCs (42)	(1) ACN; (2) 10% TCA/ ACN, 12.5% NH_3; (3) NFPA/ ACN, 12.5% NH_3; (4) Water, 1 M Na_2HPO_4 (pH 12.0)/ ACN[a]	Zorbax SB-C18 (50 × 2.1 mm, 1.8 μm)	Gradient: 1 mM NFPA[b] in 0.5% FA/ ACN:MeOH (50:50, v/v)	LC-MS/MS (ESI+)	29–81	[90]
CAP, lincomycin, MACs, 5 QNs 1 SA, 4 TCs and others (17)	Water/ Strata-X-SPE	Polar-RP Synergi (50 × 2.0 mm, 4 μm)	Gradient: 0.1%FA/0.1% FA in ACN	LC-MS/MS (ESI±)	0.13–6.7	[29]
4 MACs, 5 QNs, 4 SAs, 4 TCs (17)	100 mM Na_2EDTA (pH 4.0), Oasis HLB-SPE	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	Gradient: 0.05% FA/ MeOH	UHPLC-MS/ MS (ESI+)	0.1–1.0	[91]
12 β-lactams, 23 MACs, 8 NMZs, 16 QNs, 24 SAs, 6 TCs and others (112)	50 mM Succinate buffer (pH 5.0)/ ACN, 12.5% NH_3 to pH 6.5, water, Evolute ABN-SPE	Kinetex C18 (150 × 2.1 mm, 2.6 μm)	Gradient: 0.3% FA in Water:ACN (95:5, *v*/*v*)/0.3% FA in water:ACN (5:95, v/v)	LC-HRMS (Exactive) (ESI+)	Not provided[c]	[92]
3 lincosamides, 10 MACs, 13 QNs, 7 TCs, TRM[d] (36)	MacIlvaine buffer (Na_2EDTA) (pH 4.0)/Oasis HLB-SPE	Aqua (150 × 2.0 mm, 3.0 μm)	Gradient: 0.2%FA/0.2% FA in ACN	LC-MS/MS (ESI+)	9.4–20	[93]
2 amphenicols, 6 MACs, 4 NMZs, 5 QNs, 12 SAs, 1 TC and others (391)	Water/1% FA in ACN	Hypersil GOLD aQ C18 (100 × 2.1 mm, 1.7 μm)	Gradient: 4 mM AF in 0.1% FA/4 mM AF, 0.1% FA in MeOH	UHPLC-HRMS (Exactive) (ESI±)	10–50	[94]
2 amphenicols, 6 MACs, 3 QNs, 13 SAs and others (31)	Water (Na_2EDTA)/ QuEChERS (1% AcOH in ACN) without d-SPE	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	Gradient: 0.1% FA, 10 mM AF/ ACN	UHPLC-HRMS/MS (Q-TOF) (ESI+)	1–20	[95]
9 NMZs, 8 QNs, 10 SAs (27)	2 M HCl, water/hexane, SCX-SPE	Poroshell 120 EC-C18 (100 × 3.0 mm, 2.7 μm)	Gradient: 0.1% FA/0.1% FA in ACN	LC-MS/MS (ESI+)	0.19–2.5	[96]
CAP, 3 MACs, 7 SAs, other drugs (2) and 79 pesticides (92)	MacIlvaine buffer (pH 4.0)/ ACN, Florisil (d-SPE)	Acquity UHPLC BEH C18 (100 × 2.1 mm, 1.7 μm)	Gradient: 5 mM AF in 0.1% FA/5 mM AF, 0.1% FA in MeOH	LC-MS/MS (ESI±)	0.12–2.8	[97]

Compounds	Extraction/ clean-up	Separation		Equipment	CCβ or LOD (μg/kg)	References
		Column	Mobile phase			
3 AGs, LIN[e], 5 MACs, 6 SAs, 8 TCs (22)	Water, 2 M, HCl in MeOH, Na_4EDTA to pH 2.0/PSA (d-SPE)	Zorbax SB-C18 (100 × 2.1 mm, 3.5 μm)	Gradient: 100 mM HFBA[f]/ water/ACN	LC-MS/MS (ESI+)	7–33	[98]

[a]Four subsequent LLE steps were carried out.

[b]NFPA, nonafluoropentanoic acid (ion-pairing reagent).

[c]CCβs for permitted antibiotics (lincomycin, MACs, QNs, SAs, TCs) were provided considering a hypothetical MRL of 100 or 200 μg/kg. For banned substances (NMZs), CCβs were in the range 1.2–2.6 μg/kg.

[d]TRM, trimethoprim.

[e]LIN, lincomycin.
[f]HFBA, heptafluorobutyric acid (ion-pairing reagent).

Table 11. Multiclass confirmatory methods.

2. Honeybee diseases

Honeybees are affected by fungal, bacterial, viral (Thai Sac brood) and acarine (*Varroa*) diseases. Antibiotics are generally used to fight bacterial and fungal diseases such as American foulbrood, European foulbrood and nosemosis [3].

American foulbrood is by far the most virulent brood disease known in honeybees. The disease is caused by the spore-forming bacterium, *Paenibacillus larvae*. Larvae up to 3 days old become infected by ingesting spores that are present in their food. Spores germinate in the gut of the larva and the vegetative form of the bacteria begins to grow, taking its nourishment from the larva. Infected larvae normally die after their cell is sealed. The vegetative form of the bacterium, before to die, produces many millions of spores which are extremely resistant to desiccation and can remain viable for more than 40 years in honey and beekeeping equipment. Because of this persistence, in most countries official apiary inspectors are required to burn all infected colonies. Other countries (e.g. USA, Canada, and Argentina) allow the use of antibiotics, such as oxytetracycline and tylosin, to keep the disease in control. However, antibiotics are not a cure or a treatment of the infection since they affect only the vegetative stage of American foulbrood, inhibiting its development in the gut of the larvae. This may prevent the rapid diffusion within a colony.

European foulbrood is closely related to American foulbrood in symptomatology, and until 1906, these two diseases were not differentiated. The causative organism of European foulbrood is the bacterium *Melissococcus plutonius*, which does not produce spores, and therefore, this disease is considered less severe than American foulbrood. European foulbrood occurs primarily in spring when numbers of *M. plutonius* reach their peak. The bacterium is ingested by honey bee larvae and it replicates in mid-gut. If the bacteria out-compete the larva, the larva will die before the cell is capped. Alternatively, the bee may survive until adulthood if the larvae has sufficient food resources. Some antimicrobials, for example, oxytetracycline, have been demonstrated to be an effective treatment.

Nosemosis, caused by the fungus *Nosema apis* or *Nosema ceranae*, is historically considered the most serious disease of adult bees. Infection is acquired when spores are swallowed by bees and infect the epithelial cells of the hind gut, giving rise rapidly to large numbers of spores and impairing the digestion of pollen which shortens the life of honeybees. *N. ceranae* was originally a parasite of the Asian honeybee (*Apis cerana*), but now is widespread in some European regions, too. In recent years, the disappearance of adult honeybees, known as colony collapse disorder, has been devastating a great number of beehives worldwide. This problem has caused serious damage to apiculture and also to agricultural activities that use honeybees as pollinators. Among the possible causes of the disappearance of honeybees, nosemosis has been reported as a primary candidate.

3. Methods for the determination of drug residues in honey: sample treatment

3.1. Matrix-analyte

Sample treatment is fundamental in the residue analysis of food, since the achievement of low detection limits (some parts per billions) and suitable selectivity involves extensive purification of generally complex food matrices. The sample preparation process consists of the extraction followed by one or more purification steps. Rarely, the purification step is omitted. To decide the sample treatment strategy, main aspects have to be considered: the characteristics of both sample matrix and he physico-chemical properties of analyte(s) have to be taken into account, together with, in addition, the already developed procedures (literature searching).

Because of the hydrophilic nature of honey, frequently, the extraction coincides with the sample dissolution in pure water or in acidified aqueous solutions or in buffers. After that, besides the traditional liquid-liquid extraction (LLE) and solid-phase extraction (SPE) purifications, more recent clean-up methodologies have been applied such as quick, easy, cheap, effective, rugged and safe (QuEChERS), molecularly imprinted polymers (MIPs) and multi-walled carbon nanotubes (MWCNs). These two latter are particular kinds of SPE, whereas QuEChERS methodology is a variation of LLE, followed by a dispersive solid-phase extraction (d-SPE) step. It is important to keep in mind that, despite the proliferation of dozens of new purification approaches with various acronyms, essentially all these fall into LLE or SPE techniques. Some additional examples are microextraction by packed sorbent (MEPS), stir bar sorption extraction (SBSE), dispersive liquid-liquid microextraction (DLLME) and phase separation-based magnetic-stirring salt-induced liquid-liquid microextraction (PS-MSLM). These recent methodologies give also evidence of the current trend towards "micro", that is, towards a lower consumption of reagents and materials during the sample treatment. Less common and expensive purification systems such as turbo-flow chromatography are not here considered.

Dissociation constants (pK_as) and lipophilicity are key parameters to understand the behaviour of drugs, and therefore, to perform appropriate extraction and purification strategies, physico-chemical properties of a drug molecule are described by its pK_a(s) and its polarity pK_a (dissociation constant) is a measure of the strength of an acid or a base. It determines the

charge on a molecule at any given pH. The lipophilicity polarity is measured by the partition coefficient, P, or better, by the distribution coefficient, D, which are the key parameters to understand the behaviour of molecules, and therefore, to design appropriate purification strategies during the method development, P is the ratio of the concentration of a compound in octanol to its concentration in water P (Eq. (1)):

$$P = \frac{[\,\text{drug}]_{\text{octanol}}}{[\,\text{drug}]_{\text{water}}} \tag{1}$$

P is generally expressed as logarithm of the log P. Log P is a constant for the molecules under its neutral form, and its value is a measure of lipophilicity or hydrophobicity. On the other hand, the distribution coefficient (D, or better, its logarithm, log D) takes into account all neutral and charged forms of the molecule. Therefore, for ionizable solutes, such as drugs, the pH-dependant lipophilicity descriptor, that is, the distribution coefficient (D), is more appropriate. D is the ratio of the sum of the concentrations of all forms of the compound (ionized plus un-ionized) in each of the two phases, octanol and water, (Eq. (2)):

$$D = \frac{[\,\text{drug molecule}]_{\text{octanol}}}{[\,\text{drug molecule}]_{\text{water}} + [\,\text{drug ion}]_{\text{water}}} \tag{2}$$

Roughly, when log D < 0, the molecule is polar (hydrophilic) and vice versa. Because the charged forms hardly enter the octanol phase, this distribution varies with pH. In the pH region where the molecule is mostly unionized, log D = log P. Acids are neutral when protonated and negatively charged (ionized) when deprotonated. Bases are neutral when deprotonated and positively charged (ionized) when protonated. Therefore, the log D of a compound is strongly influenced by its acid-base dissociation constant(s), pK_a. However, log D values cannot furnish precise information about the ionization status of the compound mainly because frequently more than one acidic or basic centre can be present in its structure. Only the knowledge of the pK_as allows the understanding of the predominant forms at the various pH values. In **Figures 1–3**, the plots of log D versus pH of one representative compound per class are shown. These plots were obtained applying the MoKa® package (Molecular Discovery Ltd.) [13]. This software package is able to predict also the pK_as. Ranges of pHs increasing log D (lipophilicity) can favour RP-SPE and LLE purification strategies, which are based on the analyte transfer from a more polar medium (honey solution) to a less polar one. On the other hand, selective purifications such as ion-exchange SPE are enabled when the analytes are in their ionized form and, therefore, in pH intervals where log D values are lower (higher hydrophilicity).

3.2. Purification

Liquid-liquid extraction (LLE) is one of the first sample preparation approaches and continues to be widely used. LLE is based on the transfer of an analyte from the aqueous sample to a water-immiscible solvent based on its distribution coefficient, D. The water-immiscible solvents can be ethyl acetate, dichloromethane and chloroform. Nevertheless, some shortcomings, such as emulsion formation, the use of relatively large sample volumes and toxic organic solvents, make the traditional LLE (relatively) expensive and environmentally harmful. To avoid emulsion formation, supported liquid extraction (SLE) can be applied.

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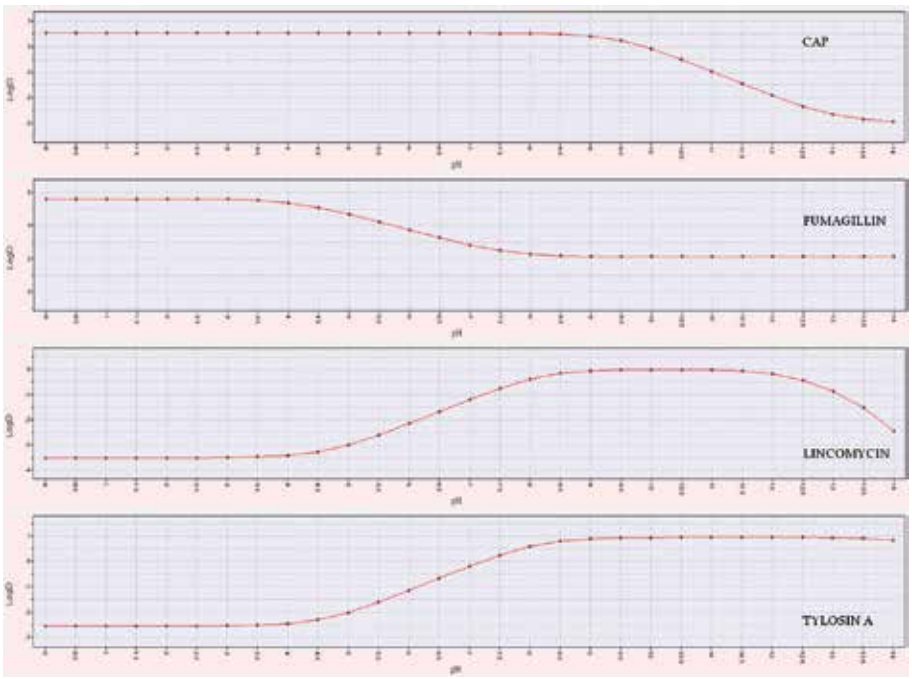

Figure 1. Log D versus pH for chloramphenicol (CAP), fumagillin, lincomycin and tylosin A (MAC).

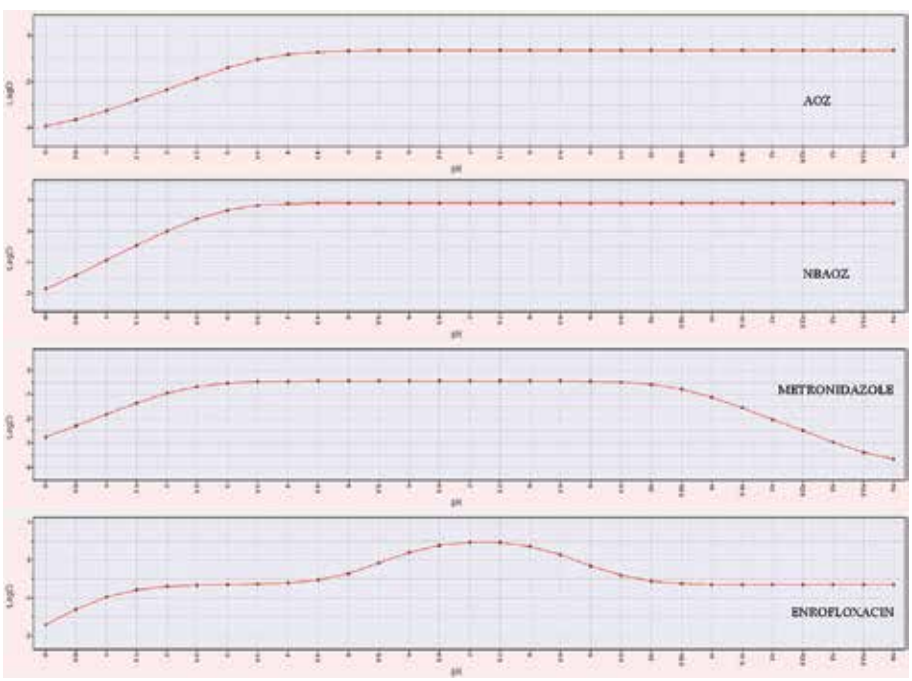

Figure 2. Log D versus pH for AOZ, derivatized AOZ (NBAOZ), metronidazole (NMZ) and enrofloxacin (QN).

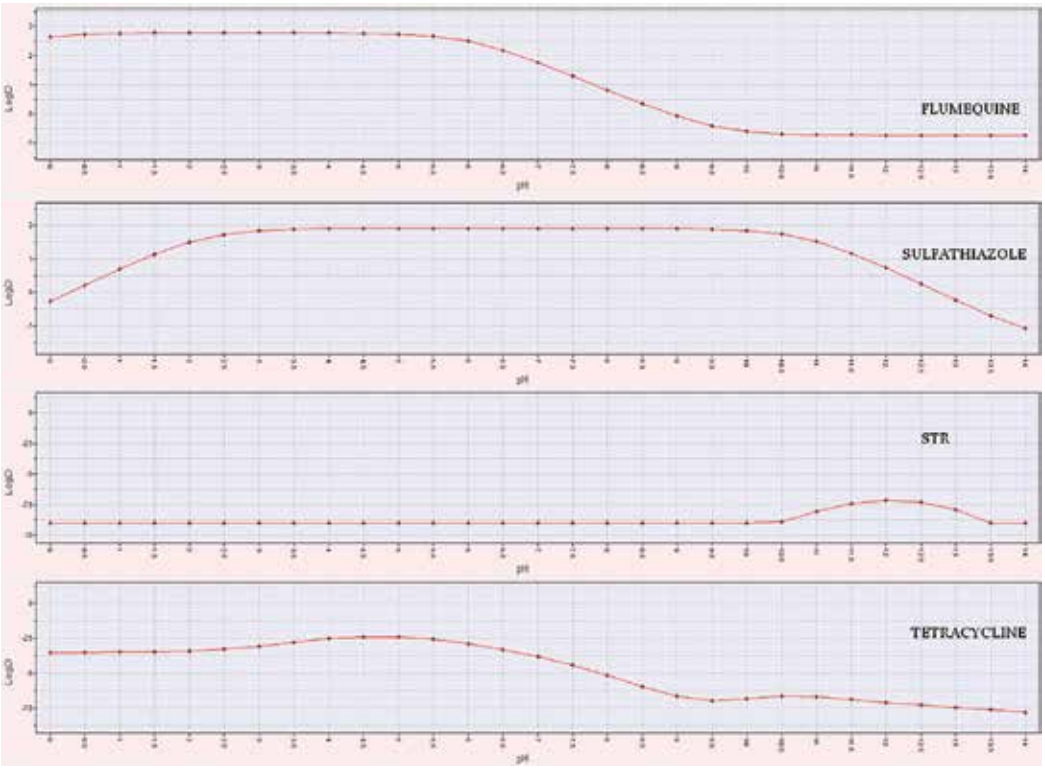

Figure 3. Log D versus pH for flumequine (QN), sulfathiazole (SA), streptomycin (STR) and tetracycline (TC).

Its principle is simple: a chemically inert, high surface area support, highly purified, graded diatomaceous earth (Extrelut®, Hydromatrix®, Celite®, etc.) serves as a stationary vehicle for the aqueous phase of the liquid-liquid extraction experiment. The aqueous-based sample (e.g. diluted honey) is added to the dry sorbent and allowed to wet the diatomaceous earth. A small volume of immiscible organic extraction solvent is then added and allowed to percolate by gravity through the supported aqueous phase. Because the aqueous sample has been widely dispersed throughout the solid support, the organic solvent has intimate contact with the thin film of aqueous phase and rapid extraction (equilibration) occurs.

Even today, probably, solid-phase extraction (SPE) is the most used sample purification tool in trace analysis. This technique was developed in the mid-1970s as an alternative to LLE. The degree of selectivity of SPE technique can be very different, depending on the attractive forces between the analytes and the functional groups on the sorbent surface. SPE sorbents are most commonly categorized by the nature of their primary interaction or retention mechanism with the analyte(s) of interest. The sorbent can interact with analytes by hydrophobic (non-polar/non-polar), hydrophilic (polar-polar, hydrogen bonding, dipole-dipole, π-π interactions) and cationic-anionic interactions. The most common SPE sorbents packing can be classified into non-polar phases (reversed phases—RP), polar phases (normal phases—NP), ion-exchange and immunoaffinity adsorbents.

Non-polar sorbents are used under RP chromatography conditions and are suitable for the extraction of hydrophobic or polar organic analytes from aqueous matrices. Accordingly, reversed phase is the most used SPE sorbent type to purify honey, which is a water-soluble matrix. These sorbents comprise alkyl silica and polymer-based materials. Alkyl silica sorbents are manufactured by bonding alkyl or aryl functional groups, such as octyl (C8), octadecyl (C18) and phenyl (Ph) to the silica surface. It should be noted that in SPE, the interactions described above are not found in pure form, but in combination. For example, C18 silica-based sorbents are non-polar sorbent, but it still possess free silanol groups, which can produce hydrophilic secondary interactions. The retention of analytes under RP conditions is due primarily to the van der Waals attractive forces between the carbon-hydrogen bonds in the analytes and the functional groups on the silica surface. The elution of adsorbed compounds is generally made by using a non-polar solvent (compared to water) to disrupt the forces that bind the compound to the sorbent. However, silica-based bond phases contain non-uncapped silanols, which can cause the strongly binding of some group of compounds (i.e. tetracyclines), and in addition, they can be used only in a limited pH range (2–8). Currently, silica materials have been more and more replaced by polymeric sorbents. The macroporous wettable hydrophilic-lipophilic balance (HLB) polymeric sorbent (divinylbenzene-N-vinylpyrrolidone) was at first introduced by Waters Company (Oasis HLB). Later, other manufacturers commercialized similar reversed-phase proprietary polymeric sorbents such as Strata-X (surface-modified styrene-divinylbenzene; Phenomenex), LiChrolut EN (highly cross-linked polystyrene-divinylbenzene; Merck, Darmstadt, Germany) and Evolute ABN (cross-linked polystyrene-divinylbenzene functionalized with oligomeric hydroxyl groups; Biotage). These cartridges have been widely applied in honey purification of almost all antibiotic classes.

The intrinsic honey characteristics undoubtedly favour the wide application of RP-SPE purification approaches since NP-SPE is more suitable to isolate a polar analyte in a mid- to non-polar matrix (acetone, chlorinated solvents, hexane, etc). The most common polar stationary phases are silica, alumina and florisil. Retention of an analyte under NP conditions is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface (hydrogen bonding and π-π interactions, among others). The passing of a solvent that disrupts the binding mechanism, usually a solvent that is more polar than the sample matrix, allows the elution of the adsorbed compounds. To the best of our knowledge, examples of NP-SPE purification applied to determination of veterinary drug residues in honey are limited to nitroimidazole family (**Table 6**). This is probably why nitroimidazoles are very polar compounds. The application of this kind of sorbents generally involves a preliminary liquid-liquid extraction step to transfer the analytes from the aqueous phase (solubilized honey) to an organic phase (non-polar matrix) which is then loaded onto the cartridge.

Due to their selectivity, ion-exchange SPE sorbents can be generally used only in single-residue or single-class procedures. These sorbents are very efficient for extraction of charged analytes, such as acidic and basic compounds, from aqueous or non-polar organic samples. Ion-exchange phases are comprised of positively (aliphatic quaternary amine, aminopropyl) or negatively (aliphatic sulphonic acid, aliphatic carboxylic acid) charged groups. Porous polymer, ion-exchange resins have a higher exchange capacity and a wider pH operating range than silica-based materials. Ion-exchange sorbents are usually classified as weak or

strong, depending on the identity of the ionic group and whether its charge is independent of the sample pH (strong ion exchanger) or can be manipulated by changing pH (weak ion exchanger). Antibiotic substances have frequently basic functional groups, and therefore, the application of both strong cation exchange and weak cation exchange has been reported also in honey, mainly for the determination of streptomycin/dihydrostreptomycin (**Table 8**) and sulphonamides (**Table 9**). Finally, the immunoaffinity chromatography is a SPE technique, based on very selective antigen-antibody interactions (immunosorbents); examples of its application to purify honey have been reported, too.

In some cases, both liquid-liquid extraction and solid-phase extraction can be used in an "opposite manner", that is, solubilizing or retaining the interfering substances rather than the analytes. An important example in antibiotic analysis is the so-called defatting to purify food extracts in water-miscible solvents: the added water-immiscible solvent (generally hexane) does not solubilize the analytes of interest, but the highly lipophilic interfering substances (fats), and therefore, it is discarded. Analogously, in the "non-retentive" SPE the sorbent has no affinity for the analytes, but for the sample contaminants. The solid phase is simply used to "filter" the sample: analyte passes through the column without being retained, while (part of) the contaminants are retained. This kind of extraction is generally applied when the analyte is highly soluble in the sample matrix (or in the dilution solvent), and therefore, it cannot be partitioned out onto a solid sorbent (retentive SPE) or an immiscible solvent (LLE).

Among the relatively modern purification approaches, it may be worthwhile to describe the QuEChERS, molecularly imprinted polymers (MIPs) and multi-walled carbon nanotubes (MWCNs) methods. The QuEChERS approach has become particularly popular for the multiresidue analysis of pesticides in various food matrices, and it generally consists of two steps: first, the homogenized sample is extracted and partitioned using an acetonitrile and salt solution ($MgSO_4$ and NaCl), and then, an aliquot of the supernatant is cleaned using a dispersive solid-phase extraction (d-SPE) technique. Dispersive SPE is a "non-retentive" SPE, because the matrix co-extractives are adsorbed onto the sorbent, while leaving analytes of interest in the solvent. In some applications of QuEChERS, the second step (d-SPE purification) can be omitted. MIP sorbents are highly cross-linked polymers with a predetermined selectivity towards a single analyte or group of structurally related analytes. This selectivity is obtained during the synthesis of the polymer by using a template molecule to form cavities with specific shape. The process usually involves initiating the polymerization of monomers in the presence of the template molecule that is extracted afterwards, thus leaving complementary cavities behind. Due to the high selectivity of these sorbents, they generally allow for lower detection limits. In recent years, multi-walled carbon nanotubes, a new kind of carbon material, have attracted much interest that is directed towards the development of solid-phase extraction adsorbents. The MWCNs were promising sorbents because of the larger specific area and the dramatic hydrophobic characteristic of the surface. The adsorption mechanisms involve weak interactions (mainly π-π stacking, van der Waals and electrostatic forces), facilitating the adsorption of analytes in a selective and reproducible manner.

To conclude, the current trends in food sample preparation involve the following issues: the miniaturization of the equipment for sample preparation (micro techniques); the decrease in the amount of sample to be analysed; the reduction in the use of organic solvents; the

development of multiclass procedures; and the development of automated methods for the preconcentration. All these strategies aim at the reduction in the employed reagents/materials and at the increase in the analysis throughput.

4. Methods for the determination of drug residues in honey: analytical techniques

Until early 2000s, LC-UV-Vis and LC-FLD were the most used equipments to detect residues in food. UV-Vis detectors measure solute analytes by their absorbance in the ultraviolet or visible region. A UV detector employs a deuterium discharge lamp (D_2 lamp) as a light source, with the wavelength of its light ranging from 190 to 380 nm. If substances are to be detected at longer wavelengths, that is, in the visible region (380–700 nm), a UV-VIS detector is used with an additional tungsten lamp (W lamp). Nowadays, photodiode arrays and DAD (semiconductor devices) have replaced UV-Vis detectors, and its use is mandatory to definitively confirm the presence of residues of permitted veterinary drugs in food [6]. A DAD detects the absorption in UV to VIS region. While a UV-VIS detector has only one sample-side light-receiving section, a DAD allows the acquisition of full wavelength spectrum at one time thanks to multiple photodiode arrays. Spectra are measured at regular intervals (one second or less) during the LC separation with continuous eluate delivery. Therefore, to identify a compound, in addition to the retention time, DAD enables the comparison between the spectrum of the authentic standard and of the analyte. It is important to underline that according to Commission Decision 2002/657/EC, only the coupling between LC and DAD (not between LC and UV-Vis) allows the definitive confirmation of residues of permitted substances in food.

Fluorescence detectors have greater sensitivity and selectivity over the UV-Vis ones. This is an advantage for the measurement of specific fluorescent species in samples; however, only about 15% of all compounds have a natural fluorescence. Compounds having specific functional groups are excited by shorter wavelength energy and emit higher wavelength radiation. This phenomenon is called fluorescence. Generally, the presence of aromatic conjugated pi-electrons produces the most intense fluorescent signal. Most unsubstituted aromatic hydrocarbons fluoresce with quantum yield increasing with the number of rings, their degree of condensation and their structural rigidity. In addition, aliphatic and alicyclic compounds with carbonyl groups and substances with highly conjugated double bonds fluoresce, but usually to a lesser extent. Among veterinary drugs, quinolones possess native fluorescence; some other antibiotic classes can be efficiently derivatized to give fluorescent compounds (e.g. sulphonamides and aminoglycosides).

For the analysis of residues in food, nowadays, LC-MS is the standard internationally accepted technology already available in most laboratories that is capable of providing structural information about the analytes. Different mass spectrometer platforms have been successfully employed for the analysis of veterinary drugs in honey [14]. Since early 2000s, triple quadrupole mass spectrometer (LC-MS/MS) platform has been introduced in routine worldwide laboratories, and at present, this MS technology is the gold standard for routine analysis of complex sample extracts. The LC-MS/MS, also known as LC-QqQ, is a tandem MS technique in which the first and third quadrupoles act as mass filters and the second, a radio-

frequency-only quadrupole, fragments the analyte through interaction with a collision gas. The most used acquisition mode is multiple reaction monitoring (MRM). Increased selectivity, improved signal-to-noise ratio (S/N), lower limits of quantitation, wider linear range and improved accuracy are some of the benefits of this technique. LC-MS/MS instrumentation tends to give better quantitative precision and improved sensitivity than alternative configurations, making it a superior choice for routine analysis of specific targeted contaminants.

An alternative to LC-MS/MS system is the coupling of liquid chromatography with high-resolution mass spectrometry (LC-HRMS). At the beginning, these analysers were mainly used for research purposes, but after 2007 they started to be applied in routine analysis, too. With HRMS analysers, full-scan spectra are continuously obtained throughout the analytical run allowing for exhaustive qualitative post-acquisition analysis. There are two technologies of high-resolution mass spectrometry: time-of-flight (TOF) and orbitrap. However, single-stage high-resolution mass spectrometry demonstrated to not be suitable for the confirmation of residues at very low concentrations in highly complex matrices such as honey. More recently, hybrid platforms have been available at the bench level such as Q-TOF and Q-Orbitrap combining a quadrupole with an accurate mass analyser. These configurations provide exceptional selectivity and sensitivity over single-stage equipment, and they are increasingly applied in residues analysis of food.

With regard to the chromatographic separation, although the coupling between gas chromatography and mass spectrometry (GC-MS) has been realized before LC-MS, gas chromatography is rarely used for the determination of antibiotics, due to their polar nature, low volatility and thermal instability. Therefore, high-performance liquid chromatography (HPLC) is the technique of choice for antibiotic analysis. Since its introduction in 1970s, HPLC progressively improved mainly thanks to the evolution of packing materials used to carry out the separation. Columns packed with 10 and 5 μm fully porous particles dominated the field for nearly thirty years (1975–2000). In 2004, a great advance in instrumentation and column technology was made achieving very significant increases in resolution, speed and sensitivity. Columns with smaller particles (sub 2-μm) and instrumentation able to deliver mobile phase at 15,000 psi (1000 bar) allowed the achievement of a new level of performance. This new step of HPLC is known as UHPLC technology. In 2007, LC columns with core-shell (superficially porous) particles were introduced. This new generation of microspheres provides the same high efficiency of sub 2-μm UHPLC totally porous particles, but with lower backpressures. The first commercially available core-shell sorbent was the Halo® from Advanced Material Technologies. Currently, the most applied core-shell columns are Kinetex® (Phenomenex), Poroshell® (Agilent), Accucore® (Thermo Fisher Scientific), Ascentis Express® (Supelco), Cortecs® (Waters) and Nucleoshell® (Macherey Nagel). Many of these have been used to determine residues in honey (see **Tables 3–11**).

5. Overview of methods for the determination of drug residues in honey

In the following paragraphs for each compound or class of compounds, an overview of the published confirmatory methods for the determination of residues in honey is given in **Tables 3–11**. Although widely applied in routine laboratories as screening methods, procedures based on bioanalytical techniques such as immunoenzymatic or receptor tests are not considered.

In **Tables 3–11**, for each reviewed procedure, the method limits (CCβs or LODs) are reported. Method limits are generally estimated by the LOD parameter, but, unfortunately, Commission Decision 2002/657/EC [6] introduced a different terminology, that is, decision limit (CCα) and detection capability (CCβ). Although the estimation of method limits is one of the most problematic topics of analytical chemistry [15], from a theoretical point of view, LOD and CCβ (for banned substances) are essentially the same parameter taking into account of both alpha-error (false-positive rate) and beta-error (false-negative rate) [16]. In the relevant column of the tables, CCβs are reported, if available, or, alternately, LODs. They are always given with a maximum of two significant figures.

The most used technique is LC-MS, in particular LC-QqQ platform (**Tables 3–1**). The need of reaching low concentrations involves a progressive decline of LC-DAD- and LC-FLD-based procedures. Methods based on LC-MS (single quadrupole) platform are sporadically described. Finally, in the last few years, high-resolution mass analysers are more and more applied. The ionisation source is almost always electrospray in positive mode (ESI+), except for chloramphenicol for which negative ionization is largely favoured (ESI−). The chromatographic separation is generally achieved in reversed-phase mode, except for aminoglycosides (streptomycin and dihydrostreptomycin) where HILIC columns are frequently applied.

5.1. Chloramphenicol (CAP)

Chloramphenicol is a potent, broad-spectrum antibiotic and a potential carcinogen and has been banned in the European Union since 1994 for use in food-producing animals. The United States and Canada, as well as many other countries, have completely banned its usage in the production of food, too. In January 2002, concerns regarding serious deficiencies of the Chinese residue control system and problems related to the use of banned substances in food-producing animals led the European Union to issue a suspension of imports of all products of animal origin from this country. Meanwhile, a growing number of rapid alert notifications related to the presence of CAP in imported honey have been issued. In beekeeping practice, this antibiotic is mainly used to fight the American foulbrood disease. In 2002, 31 cases out of 34 positive CAP honey detected by the RASFF system (**Table 2**) were from China. These findings were confirmed by Verzegnassi et al. [17] who in the same period analysed 176 raw honeys of various geographical origins, showing very extensive contamination in those of Chinese origin (29 positive samples out of 32). One year later (2003), the percentage of positive chloramphenicol honey from China fell down with only one notification. The import ban was lifted in July 2004.

In **Figure 4**, the sample preparation protocols proposed by the authors of the nine selected analytical methods listed in **Table 3** are summarized [17–25]. Using the CAF as "case study", the figure generalizes the sample purification concept, which is a modular process composed of one or more LLE and SPE steps. Generally, honey is dissolved in water or in acidic solutions due to better solubility of CAF in organic solvents at these pHs (**Figure 1**), thus enabling subsequent RP-SPE or LLE purification. Only Alechaga et al. [23] solubilize honey in an aqueous basic solution (1% NH_3), to favour the adsorption on the stationary phase (Oasis HLB) of florfenicol amine which was included in the same procedure. As explained by its name, florfenicol amine (the main metabolite of florfenicol) is a basic drug non-ionized at pHs exceeding 9. The solubilized honey is then purified with one or two clean-up steps: (a) SPE [20, 21, 23];

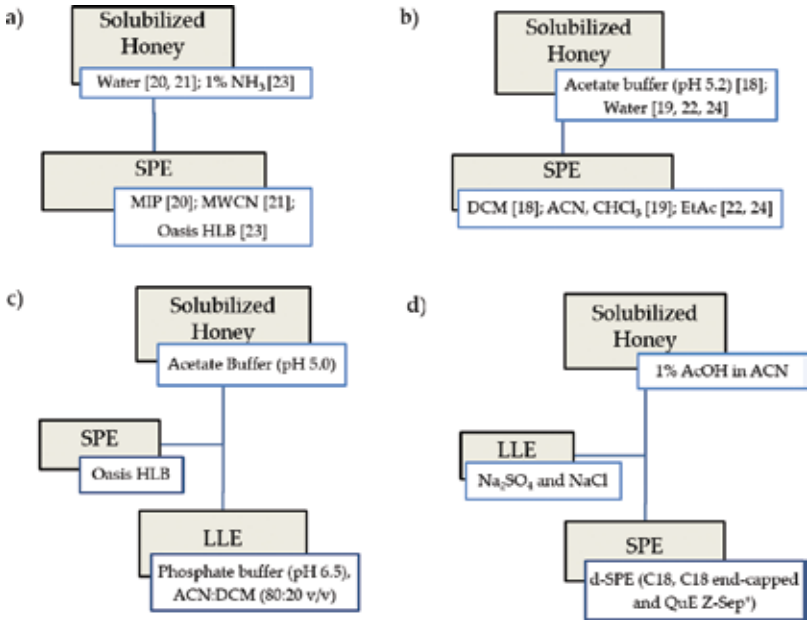

Figure 4. Sample treatment strategies for the determination of **chloramphenicol** residues in honey (**Table 3**): (a) [20, 21, 23]; (b) [18, 19, 22, 24]; (c) [17]; (d) [25].

(b) LLE [18, 19, 22, 24]; (c) SPE and LLE [17]; (d) LLE and SPE [25]. The same scheme could be realized for all the other antibiotic methods summarized in **Tables 4–11**. A complete overview of the sample preparation issues is available in "Analysis of Antibiotic Residues in Food" [26].

5.2. Fumagillin

Fumagillin is a potent amoebicidal agent with properties known since 1950s. This compound is used by apiarists to protect bees from *Nosema apis*. A few articles have reported methods for its determination. The first procedure using LC-MS technique (single quadrupole) has been developed by Nozal et al. in 2008 [27]. In 2011 and in 2015, respectively, Kanda et al. [28] and van den Heever et al. [29] published methods based on LC-MS/MS (triple quadrupole). Nozal et al. [27] and van den Heever et al. [29] applied a quite similar purification approach, solubilizing honey in water and purifying it with polymeric RP-SPE cartridge. They also reached similar LODs ranging from 1 to 4 μg/kg, depending on the honey type (botanical origin). Surprisingly, Kanda et al. [28] reported LODs of two orders of magnitude lower (0.02–0.03 μg/kg), applying QuEChERS extraction with 0.1% FA in acetonitrile followed by non-retentive WAX-SPE. These authors estimate LOD by means of the standard deviation (SD) observed in replicate experiments carried out at a low spiking level, that is, 1 μg/kg (LOD = 3 × SD). However, following the analytical chemistry detection theory, to obtain a reliable estimation of LOD, the spiking level should be close to the found LOD. Clearly, the spiking level reported by Kanda et al. [16] is not suitable, being two orders of magnitude higher than the estimated LOD. This example demonstrates the well-known issues in the estimation of method limits, which can prevent correct compari-

son among method performances. On the other hand, most of the authors do not report how the LODs are obtained, simply declaring that they are calculated according to signal-to-noise (S/N) ratio approach (LOD = 3 × S/N). Finally, it is worthy of note that among the multiclass procedures, only Lopez et al. [30] have included fumagillin within the determined analytes (**Table 11**).

5.3. Macrolides (MACs) and lincomycin

As a result of the development of resistance to oxytetracycline, in the last 15 years two macrolide antibiotics, erythromycin and tylosin, have been widely used for the prevention and treatment of apiculture diseases. Since 1970s, some studies report that tylosin was superior to sulphathiazole in the control of American foulbrood in field colonies of honeybees. In 2005 and in 2013, the US Food and Drug Administration (FDA) and Canada authorities, respectively, approved the use of tylosin in honeybees. In addition, Canada authorities fixed an MRL in honey equal to 200 μg/kg as sum of tylosin A and B (**Table 1**). The most significantly published procedures are summarized in **Table 4** [31–37]. Lincomycin belongs to the group of lincosamides, and its activity against *Paenibacillus larvae* strains has been reported. In 2012, lincomycin was approved by FDA to control tetracycline-resistant American foulbrood disease. Its structure is similar to that of macrolides, and some analytical methods determine simultaneously these substances [31, 33]. Because macrolides are unstable in acidic solution, that is, pH <4, sample extraction is generally carried out in water or in basic buffers (pH 8.0–10.5). Due to their basic nature, at these pHs the reversed-phase solid-phase extraction approach is favoured (**Figure 1**), and all procedures listed in **Table 4** purify the honey extract using silica C18 or polymeric cartridge (Oasis HLB and Strata-X).

5.4. Nitrofurans (NFs)

Nitrofurans have been used for long time in veterinary practice as antibacterial agents for treating infections caused by bacteria and protozoa. At present in Europe and other several countries, these substances are explicitly prohibited or not authorized for all food-producing animals because of their potentially carcinogenic and mutagenic effects on human health. Several studies have showed that animals rapidly metabolize nitrofurans and the in vivo stability of parent drugs is no longer than a few hours. Consequently, the detection of parent drugs in animal tissues is impractical [38]. The covalent binding of NFs with protein tissues has been proven applying the ^{14}C technique to furazolidone drug. After this observation, analytical methods able to liberate the covalently bound drugs were developed. An acidic hydrolysis followed by a derivatization step with 2-nitro-benzaldehyde (NBA) and subsequent neutralization demonstrated to be the more suitable procedure for NF residue determination. The acid hydrolysis does not release the intact drug, but a structural unit of the parent molecule. 3-Amino-2-oxazolidinone (AOZ), 5-methyl-morpholino-3-amino-2-oxazolidinone (AMOZ), semicarbazide (SEM) and 1-aminohydantoin (AHD) are the released metabolites of furazolidone, furaltadone, nitrofurazone and nitrofurantoin, respectively. It must be underlined that the derivatization with NBA of the cleaved drug metabolites is essential, since AOZ, AMOZ, SEM and AHD are very polar compounds scarcely retained on RP columns and with poor ionization properties in the electrospray interface of MS analysers. It was

thanks to the application of the hydrolysis and derivatization procedure together with the use of LC-MS/MS technique that, in the early 2000s, a large number of contaminated food samples were discovered (**Table 2**). Currently, all the methods are based on this treatment. The analysis of commercialized honey samples demonstrated that furazolidone (AOZ) is the main nitrofuran antibiotic used in apiculture [12]. Inevitably, all methods in **Table 5** apply the LC-MS techniques [39–45]. The first procedure for the determination of metabolites in honey was published by Khong et al. in 2004, using isotopic dilution [39]. Most of the procedures perform the honey solubilization directly in the derivatization mixture (usually an HCl aqueous solution with NBA) [39, 42–44], then purifying the less polar derivatized metabolites (NBAOZ, NBAMOZ, NBSEM and NBAHD). Since after derivatization the solution is neutralized (pH about 7), the LLE and RP-SPE approaches work well (log D about 1 for NBAOZ: **Figure 2**). On the other hand, a limited number of methods perform the derivatization after the first purification step [40, 41]. Tribalat et al. [40] solubilize honey in a 100 mM HCl solution and then carry out a non-retentive RP-SPE (Oasis HLB) since the non-derivatized metabolites are very polar with scarce affinity for non-polar sorbents. As shown in **Figure 2**, at pH < 2 the log D of AOZ is lower than −2. After derivatization, a second (retentive) RP-SPE to isolate NBAOZ, NBAMOZ, NBSEM and NBAHD is carried out. Analogously, Lopez et al. [41] solubilize honey in a 10% NaCl solution, and after a non-retentive RP-SPE (Oasis HLB), they derivatize the metabolites and carry out a LLE using ethyl acetate. For the first time, in 2015, Kaufmann et al. [43] applied an LC-HRMS/MS platform (LC-Q-Exactive) to identify and quantify NFs and CAP, demonstrating acceptable performances for all the four metabolites, except for SEM with CCα and CCβ higher than the fixed MRPL (1 μg/kg).

5.5. Nitroimidazoles (NMZs)

Metronidazole (MNZ), dimetridazole (DMZ), ronidazole (RNZ) and ipronidazole (IPZ) are all nitroimidazole drugs with antibiotic and antiprotozoal activity. NMZs have been traditionally used for treatment and prevention of histomoniasis and coccidiosis in poultry, trichomoniasis in cattle and dysentery in swine. Due to their mutagenicity, genotoxicity and carcinogenicity, in 1990s NMZs have been classified in Europe as prohibited substances for all food-producing species (Group A6 of Annex I of Directive 96/23 [5]). NMZs can prevent and control *Nosema apis*, and in China, these drugs have been used as a cheap alternative to fumagillin. The presence of NMZ residues in honey has been reported only in the last few years [46]. CRL Guidance Paper (2007) [4] requires methods to reach 3 μg/kg. The main published methods based on LC-MS/MS technique are listed in **Table 6** [47–51]. The 5-nitroimidazoles are known to be rapidly metabolized in animals forming the relevant hydroxy metabolites which are generally determined together with the parent drugs because they may have similar mutagenic potential. The first confirmatory procedure in honey has been published by Cronly et al. [47] in 2010, following the detection of metronidazole residues in imported honey from China and from other non-EU countries [12]. Since at pH lower than 2.5 the NMZs are ionized, the solubilization of honey in water or in buffered solution at pH 6–7 favours RP-SPE or LLE purifications (**Figure 2**). On the other hand, some authors have taken advantage of NMZ ionization in strong acidic solutions performing effective cationic-exchange purifications (SCX).

5.6. Quinolones (QNs)

QNs are widely used in veterinary practice because of their rapid effect and broad-spectrum antibacterial activity. Despite the lack of scientific data demonstrating efficacy, the application of these antibiotics in apiculture, especially in Asia, as a prophylaxis for bee diseases increased during the last few years. The first RASFF notifications for the presence of QNs in honey were reported in 2007 in Chinese products. Their use was confirmed by the frequent detection of QN residues in honey also by other control authorities, such as the US Department of Agriculture (USDA) and the Canadian Food Inspection Agency (CCFIA) [14]. To date, the only compounds found in bee products are enrofloxacin, ciprofloxacin and norfloxacin. The native fluorescence of quinolone ring has been extensively exploited to determine these antibiotics in biological fluids and food. Thanks to the high sensitivity of fluorescence detection and the lower cost of equipment compared to LC-MS, this technique is still used to detect and confirm quinolone residues in food. In **Table 7**, the most significant methods are listed [52–58]. Generally, the solubilized honey is purified by reversed-phase SPE [53, 54] or by LLE [55–57]. SPE sorbents, other than reversed-phase types, are reported in the papers published in 1998 by Rose et al. [52] and in 2011 by Yatsukawa et al. [54]. Rose et al. describe two parallel sample treatment protocols using ion-exchange solid-phase extraction: one for nine amphoteric QNs (ciprofloxacin, danofloxacin, enoxacin, enrofloxacin, lomefloxacin, marbofloxacin, norfloxacin, ofloxacin and sarafloxacin) and another for three acidic ones (flumequine, nalidixic acid and oxolinic acid). Amphoteric QNs bear both an acidic group (carboxylic acid) and a basic group (piperazinyl group), and therefore, they are positively ionized at acidic pH, enabling isolation with strong cation-exchange mechanism (SCX-SPE). On the other hand, acidic quinolones can only be neutral, or at basic pHs, they are negatively charged enabling anion-exchange purification. Yatsukawa et al. apply the classical RP-SPE (Oasis HLB) followed by metal chelate affinity chromatography (MCAC). This particular type of SPE acts via the specific chelation of quinolones with ferric ions previously bound to the stationary phase (sepharose fast flow resin). The elution is performed with a buffer (pH 4) containing Na_2EDTA. This is probably the only published application of MCAC to quinolone purification, exploiting their chelating properties. The achievable selectivity allows an efficient removal of interferences also in dark-coloured honey samples such as manuka and buckwheat [54]. On the other hand, MCAC is a well-known stationary phase to purify tetracycline antibiotics using copper (Cu^{2+}) as metal ion (see Section 5.9). Finally, in 2014, Tayeb-Cherif et al. [58] proposed a cheap and simple procedure without any sample purification (**Table 7**): the solubilized honey was just injected in the LC-FLD system. As a result, high detection capabilities (CCβ) are observed (10–100 μg/kg)

5.7. Streptomycin and dihydrostreptomycin (STR/DSTR)

Streptomycin and its derivative, dihydrostreptomycin, are aminoglycoside (AGs) antibiotics used in apiculture to protect bees against a variety of brood diseases. They are polybasic cations consisting of two or more sugars, attached to an aminocyclitol ring with glycoside linkage. Despite the fact that streptomycin is not authorized in most countries in beekeeping

practice, its use is often suggested in bee forums and in beekeeping handbooks. Residues of streptomycin and dihydrostreptomycin have been frequently detected in honey and honeybee products by the EU RASSF system (**Table 2**). Due to the lack of chromophore or fluorophore groups, the traditional absorbance or fluorescence detectors cannot be directly applied to AG determination, as shown in **Table 8** [59–68]. Fortunately, the primary amine groups in the aminoglycoside structure react with a number of derivatizing agents. Therefore, especially in the past when mass spectrometry detectors were not commonly available, methods for this antibiotic family were mainly based on liquid chromatography coupled to FLD after post-column derivatization with *o*-phthalaldehyde (OPA) or β-naphthoquinone-sulphonate (NQS). Since aminoglycosides are in polyionic form in aqueous solutions, both their extraction and preconcentration are difficult, and like the sugars of the honey, silica-based C18 sorbents are unable to retain them. The coating of silica C18 sorbents with an ion-pairing reagent such as 1-heptanesulphonic acid (AHS) was experienced to produce a temporary cation exchanger [59, 61, 62], favouring the analyte retention. In contrast, Bohm et al. [64] purify honey extracts with RP-SPE without any addition of ion-pairing reagents, probably thanks to the use of a polymeric sorbent (Oasis HLB), instead of the silica-based C18 stationary phases. Three procedures [60, 63, 66] applied weak cation-exchange extraction (WCX) to clean-up honey. In 2013, Ji et al. [65] synthesized a molecular imprinted polymer (MIP) by polymerization of methacrylic acid and ethylene glycol dimethacrylate in the presence of streptomycin as template molecule. The observed recoveries for four model compounds in honey (streptomycin, gentamicin, spectinomycin and dihydrostreptomycin) ranged from 90 to 110%. Currently, this developed MIP sorbent is commercially available and Moreno-Gonzales et al. applied it to determine aminoglycosides in honey using capillary zone electrophoresis coupled to an ion trap mass analyser [68]. Finally Wang et al. developed a home-made hydrophilic stationary phase (polyvinyl alcohol onto silica gel, PVA-Sil), which demonstrated satisfactory performances to pre-concentrate aminoglycosides in honey extracts [67].

With regard to chromatographic issues, because of their high polarity, the underivatized aminoglycosides are not sufficiently retained on standard reversed-phase HPLC columns. Therefore, there are two possible choices: (i) the addition of ion-pairing reagents such as alkyl sulphonates (e.g. sodium 1-heptansulphonic acid, AHS) or fluoropropionic acids (e.g. heptafluorobutyric acid, HFBA; pentafluoropropionic acid, PFPA) in the mobile phase and (ii) the application of HILIC (hydrophilic interaction chromatography) analytical columns, which are more compatible with MS detection since ion-pairing reagents cause strong ion suppression. HILIC is a variant of normal-phase chromatography that uses water as a strong eluent and water-miscible organic solvents like acetonitrile as organic components of the mobile phase. In **Table 8**, examples applying derivatization [59, 61], ion-pairing reagents [60, 62] and HILIC chromatography [63–67] are reported.

5.8. Sulphonamides (SAs)

As early as 1940s, sodium sulphathiazole was registered for the control of American foulbrood in United States, but its use was later banned because residues of the drug continued to be found many months after its administration. Residues of sulphadiazine, sulphadimethoxine, sulphamerazine, sulphamethazine and sulphamethoxazole have been also detected in honey

[12, 14]. Sulphonamides have good UV absorption with maxima in the range of 260–275 nm, and since the 1980s, confirmatory methods have been developed using HPLC coupled to UV detection. Moreover, after derivatization with fluorescamine, sulphonamides give fluorescence and some procedures apply LC-FLD (with pre- or post-column derivatization), reaching limits of detections (LOD/CCβ) comparable to those of LC-MS methods. In **Table 9**, some example of these applications are listed [69–78]. Since considerable amounts of SAs are bound to honey sugars, in 2000 Schwaiger and Schuch [79] demonstrated the need of an acidic hydrolysis prior to the residue analysis. This step avoids the underestimation of the actual sample contamination.

The solubility of sulphonamides in acids and alkali is conditioned by their amphoteric properties, due to the presence of an anilino amino group (pK_{a1}: 2–2.5) and of an amidic group, which contains a labile hydrogen atom with acidic properties (pK_{a2}: 6–9). Thus, sulphonamides are positively charged in acidic medium at pH <2, neutral at pH 3–6 and negatively charged at pH >6. Therefore, at one hand, exploiting their basic moiety, some procedures use strong cation exchange (SCX-SPE) to isolate sulphonamides from the acidic honey extracts [71–73, 77]. On the other hand, to successfully apply RP-SPE or LLE clean-up, some researchers buffered honey extract in the pH range about 4–6 in which the neutral form of sulphonamides prevails [70, 74–76, 78]. In this interval, the distribution coefficients (D) reach their maximum and the compound lipophilicity is enhanced, as shown in **Figure 3** for sulphathiazole.

5.9. Tetracyclines (TCs)

The efficacy of the oxytetracycline for control of European foulbrood has been widely demonstrated as early as 1950s. In honey, beyond oxytetracycline (brand name: Terramycin®), tetracycline and chlortetracycline residues have been detected, too [12, 14]. Because of their polar nature, tetracyclines have the ability to strongly bind to proteins as well as to chelate with divalent metal ions. Therefore, most extractions incorporate acidic solvents with the addition of metal chelating agents. Frequently, the extraction approaches use Na_2EDTA-McIlvaine buffer (pH = 4). Known as the "universal tetracycline extractant", McIlvaine buffer consists of citric acid and disodium hydrogen phosphate. Other common buffers used for tetracyclines extraction are oxalic acid, succinic acid and citric acid. Another challenge in tetracycline determination is their epimerization. In mildly acidic conditions (pH 2–6), epimerization occurs at position C-4. Accordingly, European Union MRLs in food are established as sum of tetracycline and its epimer, that is, tetracycline and epi-tetracycline, oxytetracycline and epi-oxytetracycline, chlortetracycline and epi-chlortetracycline [1].

As shown in **Table 10** [80–88], besides the classical reversed-phase solid-phase extraction cartridges (phenyl, Oasis HLB, Strata-X and C18), tetracyclines can be selectively purified applying a particular type of solid-phase extraction, that is, metal chelate affinity chromatography (MCAC) [82, 86]. As mentioned before for quinolones (Section 5.6), MCAC exploits tetracycline metal complexing properties to allow for additional clean-up. The sorbent (sepharose resin) is treated with aqueous copper (II) sulphate. The sample extract is then loaded onto the column and TCs are retained. The copper ions give visualization of the clean-up process: the analytes are found where the blue copper ions appear. Initially, the tetracyclines are bound to the blue copper ions on the column until disruption by an EDTA containing buffer and elution of the copper ions, EDTA and tetracyclines.

5.10. Multiclass methods

In efforts to increase the cost-effectiveness of antimicrobial residue enforcement programmes, the development of analytical methods able to detect as many contaminant compounds as possible is highly preferred. However, it is well known that one of the difficulties in the development of these procedures is the incompatibility of selective sample treatments with acceptable accuracies for a wide range of analytes. Therefore, only a generic purification protocol such as liquid-liquid extraction or reversed-phase solid-phase extraction is achievable (**Table 11**). Since generally reversed-phase sorbents provide the least selective retention mechanism when compared to normal phase or ion exchange ones, they allow the most universal solid-phase extraction approach retaining most molecules with any hydrophobic character.

There are some considerations to do before to take on multiclass methods for antibiotics: (i) the extraction of nitrofuran metabolites requires acid hydrolysis and derivatization steps that would be destructive to other analytes of interest. Therefore, this class should be extracted apart from a multiclass method to obtain satisfactory recovery and avoid degradation of acid-labile compounds; (ii) as mentioned before, highly polar compounds, such as aminoglycosides, do not perform well in multiclass methods as they are relatively insoluble in organic solvents and exhibit little or no affinity for non-polar stationary phases used in RP-LC.LC. For this reason, in **Table 11** only two papers include aminoglycosides among the determined classes adding an ion-pairing reagent in the mobile phases; (iii) in addition, in honey, the determination of sulphonamides in honey requires a preliminary hydrolysis step to measure residues bound to sugars, and therefore, also in this case, acid-sensitive antibiotics can be destroyed.

In this context, "multiclass" are procedures involving the determination of more than two drug classes. Probably, the first multiclass method in honey has been published in 2004 by Kaufmann et al. [89], reporting the determination of three antibiotic families, including sixteen sulphonamides together with three tetracyclines and flumequine, a quinolone antibiotic for which until now there is no evidence of use in apiculture. In 2008, Hammel et al. [90] developed an LC-MS/MS protocol for 42 substances including five tetracyclines, seven macrolides, three aminoglycosides, eight beta-lactams, two amphenicols and seventeen sulphonamides. Four subsequent liquid-liquid extraction steps were necessary to adequately extract all the analytes. After this paper, many confirmatory multiclass methods have been published mainly applying triple quadrupole platforms [29, 91–98]. This is in accordance with the general trend in analysis of residues in food started in the late 2000s. Although triple quadrupoles have been introduced in the mid-to late-1990s, only in recent years these equipments have improved their electronics enabling the possibility of acquiring dozens of compounds in the same chromatographic run.

6. Conclusions

The performances of an analytical method are mainly determined by the applied sample preparation and instrumental technique. The coupling honey-antibiotic (matrix-analyte) can be a "case study" to discuss the general strategies of developing methods for trace

analysis in food. It must be kept in mind that the sample preparation protocol has to start from the knowledge of the matrix composition and analyte properties (MW, pK_a, log D, etc). Moreover, the choice of the more suitable clean-up also involves the knowledge of the available methodologies, but in most of the cases the selection is limited to the SPE stationary phases. In the last years, new sorbent materials are more and more produced, enabling new possibilities for more efficient, rapid and cheap protocols. Undoubtedly, aminoglycosides and, to a lesser extent, tetracyclines are the more difficult classes to analyse. Obviously, when multiresidue or multiclass procedures are optimized, the challenge is the achievement of the best compromise among the different properties of each single-class challenging. The current trends in honey sample preparation and, more generally in food, involve the following issues: the miniaturization of the equipment for sample preparation (micro techniques); the decrease in the amount of sample to be analysed; the reduction in the use of organic solvents; and the development of multiclass procedures. All these strategies aim at the reduction in the employed reagents/materials and at the increase in the analysis throughput. The choice of the analytical equipment is less free. Today, LC-QqQ systems (triple quadrupoles) are able to solve almost each analytical problem. With regard to the analyte separation, except for aminoglycosides, reversed-phase stationary phases are generally used. Various column types (traditional, sub 2-μm and core-shell) and manufacturers have been reported in literature to determine the same analyte or class of analytes (**Tables 3–11**), but frequently the applied selection criteria are not explained or compared.

Nomenclatures

Abbreviations

2-NBA	2-Nitrobenzaldehyde
ACN	Acetonitrile
AcOH	Acetic acid
AF	Ammonium formate
AGs	Aminoglycosides
CAP	Chloramphenicol
DCM	Dichloromethane
ELISA	Enzyme-linked immunosorbent assay
EtAc	Ethylacetate
FA	Formic acid
HRMS	High-resolution mass spectrometry
LC-DAD	Liquid chromatography with diode array detector
LC-FLD	Liquid chromatography with fluorescence detection

MACs	Macrolides
MCAC	Metal chelate affinity chromatography
MeOH	Methanol
MIP	Molecular imprinted polymer
MWCN	Multi-walled carbon nanotubes
NaAc	Sodium acetate
NH_4Ac	Ammonium acetate
NFs	Nitrofurans
NMZs	Nitroimidazoles
NQS	Sodium 1,2-naphthoquinone-4-sulphonic acid
OA	Oxalic acid
PA	Orthophosphoric acid
QNs	Quinolones
QuEChERS	Quick, easy, cheap, effective, rugged and safe
SAs	Sulphonamides
SCX	Strong cation exchange
SDS	Sodium dodecyl sulphate
STR/DSTR	Streptomycin/dihydrostreptomycin
TCs	Tetracyclines
TFA	Trifluoroacetic acid
TOF	Time-of-flight
UHPLC	Ultra-high-pressure liquid chromatography

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The book Honey Analysis has 15 chapters divided into two sections: one section that is dedicated to the analysis of bioactive, physicochemical, and microbiological compounds and another that addresses techniques for the detection of residues and heavy metals. We have been able to compile a book with chapters by authors from nine countries (Brazil, Chile, Italy, Malta, New Zealand, Poland, Romania, Serbia, and Turkey) and at least three continents (South America, Europe, and Oceania). The topics discussed here are physical-chemical analysis of honey, new methods for amino acid analysis, chemical residues, heavy metals, phenolic content and bioactive components, microbiological analysis, antimicrobial activity, and honey as functional food. Also there are notions of trade and characterization of honey in these countries, presenting the reality of the local market of these countries and their perspectives so that we can know more about the techniques used as well as the importance of this activity for each country. This may facilitate the use of innovative techniques that may enable increased competitiveness and the world honey trade.

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