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MICROBIOLOGICAL AND CHEMICAL QUALITY OF SLOVAK AND EUROPEAN HONEY

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ABSTRACT

The aim of the present study was to evaluate microbiological and chemical quality of honey from Slovakia, Czech Republic and Germany. Selected microbiological and chemical parameters were determined in 12 samples of honey. Total Viable Count (TVC), coliform bacteria (CB), microscopic filamentous fungi (MFF) moisture content and free acids were determined. Plate dilution method with individual culture conditions was used for microorganisms cultivation. Moisture content was measured by refractometry and free acids content was determined by titration. The minimal value of TVC was 1.87 log CFU.g^{-1} (sample no. 11), maximal value of TVC in honey was 3.13 logCFU.g^{-1} (sample no. 7), average value of TVC was 2.52 log CFU.g^{-1} . Two samples were in accordance with Codex Alimentaius of SR (2009). Samples of honey were negative for coliform bacteria count. Four samples were negative for microscopic fungi count (sampes no. 2, 8, 9 and 11). Maximal value of microscopic fungi was 2.18 log CFU.g^{-1} in sample no. 5. Average value of microscopic fungi was 1.07 log CFU.g^{-1}. The moisture content values ranged from 16.6 % (sample no. 1) to 20.6 % (no. 3). Sample no. 3 was not in accordance with requirements of Council Directive 2001/110. Average value of moisture content was 18.3 %. The minimal value of free acids was 12 meq.kg^{-1}, maximal value was 42 meq.kg^{-1}. The average value of free acids was 28.9 meq.kg^{-1}.

Keywords: honey, total viable count, coliform bacteria, moisture content, free acids

INTRODUCTION

The quality of honey is mainly determined by its sensorial, chemical, physical and microbiological characteristics. Internationally, honey quality criteria are specified in Regulatory Standards, compiled in a Codex Alimentarius standard which at present is under revision (**Bogdanov, 1999**).
The Codex Alimentarius Standard for honey quality includes several chemical and physical parameters, comprising moisture content, mineral content, acidity, hydroxymethylfurfural (HMF) content, diastase activity, apparent sugar content, and water insoluble solids content. These analyses help the food analyst to determine the "chemical" quality of the honeys analyzed. Moreover, **Devillers *et al.* (2004)** suggest that they may be used in association with multivariate analyses to assign floral origin. These authors reported 100 % good predictions by analyses of conductivity, pH, free acidity and percentages of fructose, glucose and raffinose as variables for the principal component analysis.
Honey is the most ancient sweetener used by mankind, appreciated throughout the word, embraced by religious and cultural beliefs and today considered not only a food sources, but also a homeopathic treatment alternative for wounds, burns, oral healthcare and even a potential help in cancer treatment (**Lay-Flurrie, 2008** and **Bardy *et al.*, 2008**). It is a super saturated sugar solution characterized by a low water activity to support microbial growth (**Malika *et al.*, 2004**). The natural acidity of this product, the low protein content and the high viscosity, that limit the atmospheric oxygen penetration, are particularly stressing for several microorganisms.
The microbes of concern in honey are fungi, yeasts and spore-forming bacteria. Fungi and yeasts are responsible for honey fermentation when the moisture content is high (i.e., above 21%). *Penicillium* and *Mucor* are microorganisms usually found in honey. Moreover, the presence of strains of *Bettsya alvei*, *Acosphaera apis* and *Acosphaera major* may be indicative of bad bee-hive management practices. On the other hand, strains of *Saccharomyces*, *Schizosaccharomyces* and *Torula* predominate among yeasts (**Migdal et al., 2000**). Bacterial spores, particularly those of the *Bacillus* and *Clostridium* genus, are regularly found in honey. Sulfite-reducing *Clostridium* is an indicator organism, whose presence in honey provides evidence of contamination or pollution (**Collins *et al.*, 1999**). Spores of *C. botulinum* may be found in honey, usually at low levels (**Monetto *et al.*, 1999**). The presence of spores of *Clostridium* is especially dangerous for infants and small children (**Centorbi *et al.*, 1999**). Infant botulism is mainly caused by the consumption of honey contaminated with *C. botulinum*.
The aim of this article was to determine selected microbiological and chemical parameters of Slovak and European honey.

MATERIAL AND METHODS

The aim of this article was to evaluate microbiological and chemical parameters of honey. There were analyzed 12 samples of honey from Slovakia (samples no. 1-6), Czech Republic (samples no. 7-9) and Germany (samples no. 10-12).
The total viable count (TVC), coliform bacteria (CB) and microscopic filamentous fungi (MFF) were evaluated from microbiological parameters. The values of TVC and CB were compared with requirements of Codex Alimentarius of Slovak Republic (2009).
The moisture content and free acids were evaluated with requirements of Council Directive 2001/110.

Determination of CFU counts

Plate diluting method was applied for quantitative CFUcounts of respective groups of microorganisms in 1 g of honey. Gelatinous nutritive substrate in petri dishes was inoculated with 1 ml of honey samples by flushing and on surface in three replications.

Dilution of the samples

Basic dilution (10^{-1}) was prepared as follows: 5 g of honey content was added to the test tube containing 45 ml of distilled water. Dilution plating method was used to determine the microorganisms. For microorganism cultivation three types

of cultivating mediums were used, to segregate individual microorganism groups. Plate count agar was used for CFU segregation of TVC (incubation 48-72 h at 30 °C, aerobic cultivation method), violet red bile agar was used for CFU segregation of CB (incubation 24 h at 37 °C, aerobic cultivation method), chloramfenicol yeast glucose agar was used for CFU segregation MF (incubation 5-7 days at 25 °C, aerobic cultivation method).

Determination of moisture content by refractometry

The moisture content in honey was determined with a refractometer. Refraction of light was measured in the layer of honey. Moisture content was measured at temperature 20 °C. A drop of honey was applied between the prisms of the refractometer. Line forming the interface of light and dark areas was compared with scales of the refractometer.

Determination of free acids in honey

Free acidity was determined by titration with 0.05 mol.L^{-1} NaOH to pH 8.3. Honey samples were homogenised in a water bath and filtered through gauze, prior to analysis. Ten grams of honey were then dissolved in 75 mL of distilled water, and alcoholic solution of phenolphthalein added. The solution was titrated with 0.05 mol.L^{-1} NaOH. Acidity was determined as 10 times the volume of NaOH used in titration. The results were expressed as milliequivalent of acid per kg of honey.

Statistical Analysis

Mathematical and statistical analyses (arithmetic mean, standard deviation, standard error, coefficient of variation) were performed using the program system Statgraffic.

RESULTS AND DISCUSSION

The Codex Alimentarius determines the maximal limit of total viable counts for honey 10^2 CFU.g^{-1} (2,00 log CFU.g^{-1}), the maximal limit of coliform bacteria is 10^2 for 1 samples from five analyzed samples. **Council Directive 2001/110/EC determines the maximal limit for moisture content in honey not more than 20 % and maximal limit for free acid not more than** 50 milli-equivalents acid per 1 000 grammes.

Determination of total viable counts

The microbes of concern in honey are primarily yeast and spore-forming bacteria. Total plate counts from honey samples can very from zero to tens of thousands per gram for no apparent reason. Most samples of honey contain detectable levels of yeasts. Although yeast counts in many honey samples are below 100 colony forming units per gram (CFU.g^{-1}), yeasts can grow in honey in very high numbers. Standard industry practices control yeast growth (**Kačániová *et al.*, 2004**).

Table 1 Determination of total viable counts (TVC)

No.	TVC	
	CFU.g^{-1}	**log CFU.g^{-1}**
1	$8.9.10^1$	1.94
2	$1.09.\ 10^2$	2.03
3	$2.18.\ 10^2$	2.33
4	$1.22.\ 10^3$	3.08
5	$4.81.\ 10^2$	2.68
6	$5.63.\ 10^2$	2.75
7	$1.38.\ 10^3$	3.13
8	$7.54.\ 10^2$	2.87
9	$2.0.\ 10^2$	2.30
10	$4.77.\ 10^2$	2.67
11	$7.5.10^1$	1.87
12	$3.31.\ 10^2$	2.51

Results of our experiments showed the presence of total viable count (TVC) in amount from 1.87 log CFU.g^{-1} ($7.5.10^1$ CFU.g^{-1}) in sample no. 11 to 3.13 log CFU.g^{-1} ($1.38.\ 10^3$ CFU.g^{-1}) in sample no. 7 (Tab. 1). Average value of TVC was 2.52 log CFU.g^{-1} (Tab. 6) Only two samples (no. 1 and 11) were in accordance with Codex Alimentarius of Slovak Republic (2009).

Sinacori *et al.* (2014) found, that 35 microbial species associated with honeys and honeydew honeys were identified in their study, confirming that the stressing conditions of honey are highly selective. Thus, this matrix can be considered as a source of microorganisms useful to act in suboptimal conditions, e.g. to carry out the transformation of wastes from the food industry characterized by high concentration of sugars (e.g. molasses) in order to reduce the volume of untreated waste bulks and to valorise these substrates to obtain microbial metabolites with different applications.

Determination of CB

Sinacori *et al.* (2014) isolated and purified a total of 464 pure cultures from nectar and honey of different geographical and botanical origin. All cultures were subjected to a preliminary microscopic inspection and separated in three main groups: 423 rod shaped, Gram-positive, catalase positive, spore forming bacteria considered as presumptive *Bacillus* spp.; two rod shaped, Gram-negative, catalase-positive bacteria; 39 coccus shaped, Gram-positive, catalase negative bacteria considered as presumptive LAB. Due to the limited number of isolates and their different isolation sample, the Gram-negative isolates were subjected to the 16S rRNA gene sequencing without any differentiation at strain level: both isolates were identified as *Klebsiella pneumonia*.

Table 2 Determination of coliform bacteria (CB) in honey

No.	CB	
	CFU.g^{-1}	**log CFU.g^{-1}**
1	< 10	< 1
2	< 10	< 1
3	< 10	< 1
4	< 10	< 1
5	< 10	< 1
6	< 10	< 1
7	< 10	< 1
8	< 10	< 1
9	< 10	< 1
10	< 10	< 1
11	< 10	< 1
12	< 10	< 1

All samples were negative for coliform bacteria count, so they were in accordance with Codex Alimentarius of Slovak Republic (2009) (Tab. 2). Seven groups of SFB (spore forming bacteria) were obtained and they included several species within *Bacillus* genus and *P. polymyxa*. *B. amyloliquefaciens* resulted the species with the highest number of isolates. Several studies reported the isolation of *Bacillus* species in honeys (**Iurlina and Fritz, 2005 and Alippi and Reynaldi, 2006**).

Determination of microscopic fungi

Filamentous fungi, on the basis of macroscopic, microscopic and molecular analysis were divided into 17 groups consisting of eight different genera (*Alternaria*, *Arthrinium*, *Aspergillus*, *Chaetonium*, *Cladosporium*, *Daldinia*, *Penicillium* and *Emericella*) with the prevalence of the species *Pe. corylophilum* and *As. niger* (50 % and 32 % of the samples, respectively). *Penicillium*, *Cladosporium*, *Alternaria* and *Aspergillus* genera are considered common contaminants of honey (**Nasser, 2004**), while the other species have not been reported yet. In particular, with the exception of *Da. concentrica*, a wood saprophyte fungus (**Boddy *et al.*, 1985**), the other species belong to genera known as fungal allergens and mycotoxin producers (**Griessler *et al.*, 2010** and **Moss, 2002**).

Table 3 Determination of microscopic filamentous fungi (MFF) in honey

No.	MFF	
	log CFU.g^{-1}	CFU.g^{-1}
1	$1.36.\ 10^1$	1.11
2	< 10	< 1
3	$5.90.\ 10^1$	1.77
4	$7.27.\ 10^1$	1.85
5	$1.54.\ 10^2$	2.18
6	$1.36.\ 10^1$	1.11
7	$4.09.\ 10^1$	1.60
8	$2.72.\ 10^1$	1.43
9	< 10	< 1
10	$7.72.\ 10^1$	1.88
11	< 10	< 1
12	< 10	< 1

Results of our experiments showed, that four samples were negative for microscopic fungi count (sampes no. 2, 8, 9 and 11). The maximum value of microscopic fungi was 2.18 log CFU.g^{-1} ($1.54.\ 10^2$ CFU.g^{-1}) in sample no. 5 (Tab. 3). Average value of microscopic fungi was 1.07 log CFU.g^{-1} (Tab. 6).

Determination of moisture content

The water content of honey depends on various factors, for example: the harvesting season, the degree of maturity reached in the hive, and environmental factors (**Acquarone *et al*., 2007**).

Table 4 Determination of moisture content in honey

Sample	Refractive Index	Moisture content (%)
1	1.4946	16.6
2	1.4850	16.8
3	1.4885	20.6
4	1.4935	19.4
5	1.4890	17.2
6	1.4890	19.0
7	1.4890	19.0
8	1.4870	19.8
9	1.4930	17.4
10	1.4940	17.0
11	1.4885	19.2
12	1.4925	17.6

Moisture content in the samples of honey ranged from 16.6 % (sample no. 1) to 20.6 % (no. 3) (Tab. 4). Only sample no. 3 was not in accordance with requirements of Council Directive 2001/110. Average value of moisture content was 18.3 % (Tab. 6).

Anupama *et al*. (2003) reported moisture content varying from 17 % to 22.6 %, acidity expressed as formic acid from 0.03 to 0.15 and pH from 3.62 to 5.46 for commercial Indian honeys. **Da Azeredo *et al.* (2003)** for monofloral and heterofloral honeys have obtained values below 20 % for moisture content. In heterofloral honeys moisture varied from 18.59 % to 19.25 %. Besides, the range of acidity values varied from 28.2 to 39.5 meq.kg^{-1} being higher than the range obtained in our study for honeys from both apiaries and commerce. pH values ranged from 3.20 to 3.84.

Determination of free acids

The free acidity of honey may be explained by taking into account the presence of organic acids in equilibrium with their corresponding lactones, or internal esters, and some inorganic ions, such as phosphate (**Finola *et al*., 2007**).

Table 5 Determination of free acids in honey

Sample	Free acids (meq.kg^{-1})
1	32
2	19
3	40
4	25
5	12
6	23
7	38
8	24
9	32
10	38
11	42
12	22

Free acid in sample of honeys ranged from 12 (sample no. 5) to 42 milli-equivalents acid per 1 000 grammes (Tab. 5). All samples were in accordance with requirements of Council Directive 2001/110. The average value of free acids was 28.9 meq.kg^{-1} (Tab. 6).

Table 6 Basic statistical characteristics of honey parameters

Indicator	n	x	s	V_k%
TVC	5	2.52	0.42	16.6
MFF	5	1.07	0.85	79.43
Moisture content	5	18.3	1.34	7.32
Free acids	5	28.9	9.46	32.72

n – number of samples, x – arithmetic average, s – standard deviation, v_k – coefficient of variation (%), TVC – total viable count, MFF – microscopic filamentous fungi

Yücel *et al.* (2013) found, that the lowest mean value for the free acidity was 21.23 meq.kg^{-1} in the calluna honeys while the highest mean value for the free acidity was 30.51 meq.kg^{-1} in the pinus honeys. Variation in free acidity among different honeys can be explained by floral origin, the presence of organic acids or some inorganic ions. Free acidity values of all honeys were within the limits (lower than 50 meq.kg^{-1}).

CONCLUSION

In this work, several chemical parameters (moisture content, free acids) and microbiological parameters have been determined in 12 samples of honeys from Slovakia, Czech Republic and Germany. The values of CB, free acids and moisture content were in the range of approved limits. The values of TVC were in accordance with legislation only in two samples. Honey has two sources of contamination with microorganisms: primary sources include pollen, the digestive tracts of honey bees, dust, air, soil and nectar; secondary sources are those arising from honey manipulation by people. This contamination can be controlled by good manufacturing practices.

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