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STUDY REGARDING THE HONEY CONTAMINATION DEGREE ASSESSED IN A SPECIALIZED PRODUCTION UNIT

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Abstract

Honey represents a pleasant, nourishing food, with great biological and calorical value (315 kcal/100 g), easily digestible. It possesses real bactericidal properties, due to inhibin content. Examination of honey is necessary in order to assess its quality and purity, as well as to identify forbidden substances. The study was conducted in a processing unit which markets honey in the European Community. This study aimed to evaluate the contamination degree of the commercialised honey. In the summer of 2019, 3 batches of honey were analyzed (2 acacia honey batches containing 15 samples each and 1 batch containing 15 samples of polyfloral honey). Laboratory assessments were focused on determination of nitroimidazoles residues (metronidazole, dimetridazole, ronidazole), tetracyclines (oxytetracycline, tetracycline, chlortetracycline, doxycycline, demeclocycline, methacycline, minocycline), macrolides (clindamycin, erythromycin, josamycin, kitasamycin, lincomycin, oleandomycin, spiramycin, mirosamycin,tilmicosin, trimethoprim, tylosin), nitrofuran metabolites, chloramphenicol, streptomycin, dihydrostreptomycin, sulfonamides, trimethoprim, glyphosate. The methods used in the research were HPLC, LC-MS/MS, ELISA. The final results of the study that considered the a forementioned batches, sampled from various local beekeepers, proved that antimicrobial drug residues were in accordance with the national and international regulations, permitting marketing without restrictions.

Key words*: antibiotics residues, chloramphenicol, honey, streptomycin.*

INTRODUCTION

Honey represents the main product of beekeeping, being the result of nectar or manna bee processing and its storage in the honeycomb cells. Honey produced by bees exclusively from other raw materials than the one they naturally harvest, does not get into the frame of honey (Doliș, 2009). Honey is a natural food obtained in conventional or ecological systems, in units which follow the food safety principles, composed mainly of sugars and other constituents: enzymes, amino acids, organic acids, carotenoids, vitamins, minerals and aromatic substances (Petcu, 2006; Crivineanu et al., 2011; Tănăsoiu et al., 2014; Dobre, 2016; Dobre, 2017; Tăpăloagă et al., 2017; Tăpăloagă, 2018; Tudoreanu et al., 2012). It is rich in flavonoids and phenolic acids that act as natural antioxidants, being a beneficial product for consumers (Alqarni et al., 2012; Tănăsoiu et al., 2015; Șapcaliu et al., 2017; Tamas-Krumpe, 2019).

In terms of food safety, honey has to be free of chemical, toxic and carcinogenic contaminants, especially pesticides and antibiotics (Orso et al., 2015; Murariu et al., 2019; Murariu et al., 2019). The most common and important contaminations of honey are done directly (treatments applied in the hive) and indirectly (contaminants that come from the agriculture and the environment) (Mărghitaș et al., 2010). Chemical contaminants have different origin: environmental pollutants (heavy metals), chemicals used in agriculture (pesticides), toxic contamination substances and those formed during processing and storage stages (disinfectants, detergents and mycotoxin, chemicals which could migrate from packagings or packaging systems), direct treatment of bees against bacterial diseases of the bee brood, like *American foulbrood* or *European foulbrood* (Petcu, 2104a; Petcu et al., 2014b; Tofană, 2011).

Sulfonamides, tetracyclines, nitrofurans and macrolides are used by beekepers for

preventing and controlling honeybee diseases. Consequently, it is possible that antibiotics residues from honey may be the result of treatments carried out by beekeepers. The treatment of bees with antibiotics is prohibited in the European Union (EU), significant progress being made in the EU risk assessment legislation (Barganska et al., 2011).

Starting with 2000, for the EU food and products intended for marketing, it became necessary to establish a maximum residue limit (M.R.L.), paying more attention to the negative effects produced by the residues or their metabolites that are found in products intended for human consumption. For this purpose, analytical methodologies were developed for identification and quantification of these compounds (Lazăr et al., 2006). Techniques for extraction and purification of antibiotics from animal origin samples (including honey) include some forms of liquid-liquid extraction (L.L.E.) or solid phase extraction (S.P.E.). The most used technique for drug extraction is liquid-liquid chromatography. H.P.L.C. method (High Perfomance Liquid Cromatography) is the most extensive chromatographic method used for the antibiotic's analysis (Burian, 2011; Vlaic et al., 2018).

The major classes of antibiotics present in honey are: beta-lactams, amphenicols, tetracyclines, macrolides, aminoglycosides and fluoroquinolones. **Beta-lactams** are antibiotics used to treat bacterial infections, altering bacterial cell wall biosynthesis; for example: penicillin, ampicillin, cloxacillin, amoxicillin (Sapna et al., 2010). **Amphenicols** blocks the enzyme peptidyl transferase on the 50S ribosome. The most used are: thiamphenicol, florfenicol, chloramphenicol. Chloramphenicol is an antimicrobial with a carcinogenic potential and an unaccepted substance to be used on animals intended for human consumption, including beekeeping (Orso et al., 2015). **Tetracyclines** are used for the bacterial diseases' treatment of the bee brood; for example: oxytetracycline, chlortetracycline, tetracycline. The action spectrum resembles to the one of chloramphenicol. **Macrolides** include about 40 antibiotics, among which the most known are erythromycin, tylosin, oleandomycin and spiramycin. There are two groups: macrolides with 14 carbon atoms (erythromycin, oleandomycin) and macrolides with 16 carbon atoms (tylosin, spiramycin) (Mărghitaș et al., 2010). The most known **aminoglycosides** are gentamicin, lincomycin, neomycin and streptomycin. The polar nature of these macrolides makes it difficult to isolate them from the samples and determine their chromatography (Barganska et al., 2011). **Fluoroquinolones** are used as growth promoters; for example: ciprofloxacin, enrofloxacin, norfloxacin. **Organochlorine pesticides** are highly toxic chemical substances used in agriculture to destroy pests. The presence of pesticide residues in honey has needed the establishment of monitoring programs to determine the human exposure. Many studies have shown that organochlorine pesticides accumulate in plants from polluted soil and can enter the food chain not only through fat products but also through non-fat poducts, such as honey (Panseri et al., 2014).

Organophosphorus compounds have been used as pesticides for almost five decades. They continue to be used as insecticides, helminthicides, ascaricides, nematocides and to a lesser extent as fungicides and herbicides. Although they have been and continue to be extremely useful in combating agricultural pests around the world, their widespread use had led to numerous poisonings, even with human victims. The primary acute toxicity to mammals associated with exposure to organophosphorus pesticides results from the acetylcholinesterase enzyme inhibition (Sultatos, 2009).

In Europe, other more commercial products are used by beekeepers to control varroosis: **amitraz**, coumaphos, fluvalinate and thymol (Faucon et al., 1995). *Varroa destructor* is a hematophagus ectoparasite of bees and it is considered to be a major cause of bee colonies loss in Europe and North America (Surlis et al., 2018).

MATERIALS AND METHODS

In this paper the aim was to evaluate the contamination degree of honey sourced from beekeepers from the centre and Southern Romania in order to form a large and homogeneous batch). The analyzes were performed in a laboratory, external of the

processing unit. The study was conducted in the summer of 2019, on 3 batches of honey, collected by a local processor. The first batch consisted of 15 acacia honey samples from different beekeepers, analyzed before the honey homogenization process, the second one also consists of the same number and type of honey samples from other beekeepers from Romania, analyzed after the homogenization process, and the third lot consists of 15 polyfloral honey, each sample being harvasted from different beekeepers and .

The Nitroimidazole residues determination (metronidazole, dimetridazole, ronidazole) was carried out using the quantitative LC-MS/MS method (liquid chromatography-tandem mass spectrometry) (European regulation 37/2010/UE). Over the years, the LC-MS systems suffered significant changes, starting from simple analyses and reaching very accurate qualitative and quantitative analyses (Burian V., 2011). According to 470/2009/CE and 37/2010/CE regulations, the use of antibiotics in beekeeping is not allowed. The quantification limit of the method for metronidazole is 0.5 µg/kg, for dimetridazole is 2.5 µg/kg and for ronidazole is 0.5 µg/kg.

The tetracycline residue determination (oxytetracycline, tetracycline, doxycycline, chlortetracycline, demeclocycline, methacycline, minocycline) was conducted using the quantitative LC-MS/MS method. The quantification limit of the method is of 2 ppb, and according to the (EC) No 470/2009/CE and (EC) No 37/2010/UE Regulations, the use of antibiotics in beekeeping is not allowed.

The macrolide residue determination (clindamycin, erythromycin, josamycin, kitasamycin, lincomycin, oleandomycin, spiramycin, mirosamycin, tilmicosin, trimethoprim, tylosin) was carried out using the LC-MS/MS method. For these, there is no legal limit, because the use of antibiotics in beekeeping is not allowed. The quantification limit of this method is 2 ppb (Regulation (EC) No 37/2010/UE).

The Nitrofuran metabolites determination (semicarbazide from nitrofurazone, AOZ from furazolidone, AHD from nitrofurantoin) was conducted using the LC-MS/MS method. These substances are prohibited according to the Regulation (EC) No 37/2010/UE. The quantification limit of the method used is 1 µg/kg (Regulation (EC) No 2003/181/CE).

The chloramphenicol determination was conducted using the ELISA method. This is an officialy appoved method. In accordance with Regulation (EC) No 2002/657/CE, up to 5% false negative results may occur. Chloramphenicol is a prohibited substance according to Regulation (EC) No 37/2010/UE. The quantification limit of the sample is 0.1 ppb (Regulation (EC) No 2003/181/CE).

The streptomycin and dihydrostreptomycin residues detection were carried out using the LC-MS/MS method. For these, there is no legal limit, because the use of antibiotics in beekeeping is not allowed. The quantification limit of this method is 2 ppb (Regulation (EC) No 37/2010/UE).

The sulfonamides and trimethoprim detection was made using the LC-MS/MS method. There were determined: sulfadimethoxine, sulfaquinoxaline, sulfamethizole, sulfachlorpyridazine, sulfamoxole, sulfadoxine, sulfasalazine, sulfabenzamide, sulfaguanidine, sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamethazine, sulfamethoxypyridazine, sulfamethoxazole, trimethoprim, sulfamonomethoxine, sulfaclozine, sulfisoxazole, succinylsulfathiazole, sulfaphenazole, sulfisozole, sulfisomidine. The quantification limit of the method is between 0.5-2 µg/kg. For these, there is no legal limit, because the use of antibiotics in beekeeping is not allowed (Regulation (EC) No 37/2010/UE).

The Glyphosate residues determination was conducted using LC-MS/MS method. The quantification limit of this method is 0.010 mg/kg, the maximum residue level allowed is 0.050 mg/kg (Regulation (EC) No 369/2005/UE).

RESULTS AND DISCUSSIONS

Results and discussions regarding the contamination degree of the lot 1

The analysis of the 15 acacia honey samples, which form the first batch of the present study, the following results were obtained:

Results and discussion regarding the nitroimidazole residues determination: following the method used, LC-MS/MS, for the 15 acacia honey samples, there were obtained values below the limit of quantification, respectively: metronidazole < 0.5 μg/kg, dimetridazole < 2.5 μg/kg, ronidazole < 0.5 μg/kg. Considering the limit of quantification indicated above, this result was in accordance with Regulation (EC) No 37/2010/UE.

Results and discussion regarding the tetracycline residues determinantion: regarding the results obtained from the analysis of the acacia honey samples by the LC-MS/MS method, the values obtained are below the limit of quantification, respectively < 2 μg/kg. Considering the limit, the result was in accordance with Regulation (EC) No 37/2010/UE.

Results and discussion regarding the macrolide residues determination: following the test of the 15 acacia honey samples by the LC-MS/MS method, the results are below the limit of quantification (< 2 μg/kg). Therefore, the result was in accordance with Regulation (EC) No 37/2010/UE.

Results and discussion regarding the nitrofuran metabolites determination: the results obtained by LC-MS/MS testing of the 15 acacia honey samples are below the limit of quantification, respectively (< 1 μg/kg). The result was in accordance with the Regulation (EC) No 37/2010/UE.

Results and discussion regarding the chloramphenicol determination: following the analysis of the 15 acacia honey samples by the ELISA method, the result obtained is bellow the quantification limit of the method (< 0.1 μg/kg). Considering the limit, the result was in accordance with Regulation (EC) No 37/2010/UE.

Results and discussion regarding the streptomycin and dihydrostreptomycin residues detection: the results obtained from the analysis of the acacia honey samples by the LC-MS/MS method regarding the detection of streptomycin and dihydrostreptomycin residues, are below the limit of quantification (2 ppb). Considering the limit, the result was in accordance with Regulation (EC) No 37/2010/UE (regarding the residues of pharmacologically active substances in food products of animal origin).

Results and discussion regarding the sulfonamides and trimethoprim detection: regarding the analysis of the acacia honey samples by the LC-MS/MS method, the following average results were obtained (Table 1):

Table 1. Results and discussion regarding the sulfonamides and trimethoprim detection

Analyzed parameter in μg/kg	LOQ*	Result
Sulfadimethoxine	0.5	n.n. **
Sulfaquinoxaline	0.5	n.n.
Sulfamethizole	1	n.n.
Sulfachlorpyridazine	2	n.n.
Sulfamoxole	1	n.n.
Sulfadoxine	0.5	n.n.
Sulfasalazine	2	n.n.
Sulfabenzamide	0.5	n.n.
Sulfaguanidine	2	n.n.
Sulfanilamide	2	n.n.
Sulfacetamide	2	n.n.
Sulfadiazine	1	n.n.
Sulfathiazole	0.5	n.n.
Sulfapyridine	1	n.n.
Sulfamerazine	1	n.n.
Sulfameter	1	n.n.
Sulfamethazine	1	n.n.
Sulfamethoxypyridazine	0.5	n.n.
Sulfamethoxazole	1	n.n.
Trimethoprim	0.5	n.n.
Sulfamonomethoxine	0.5	n.n.
Sulfaclozine	2	n.n.
Sulfisoxazole	1	n.n.
Succinylsulfathiazole	2	n.n.
Sulfaphenazole	2	n.n.
Sulfisozole	2	n.n.
Sulfisomidine	1	n.n.

*LOQ = limit of quantification;

**n.n. = below the limit of quantification.

Considering the limit, the result was in accordance with Regulation (EC) No 37/2010/UE.n

All the results obtained from the analysis of the batch1 (Table 2) werein accordance with Regulation (EC) No 37/2010/UE.

Table 2. The obtained results regarding the contamination degree of batch 1 of acacia honey

Performed analyses	Result	Allowed limit
β-γ-amylase activity	2.9 U/kg	Max. 5 U/kg
Nitroimidazoles	n.n.** (0.5-2.5 µg/kg)	MRL*
Tetracyclines	n.n. (2 µg/kg)	MRL
Macrolides	n.n. (2 µg/kg)	MRL
Nitrofuran metabolites	n.n. (1 µg/kg)	MRL
Chloramphenicol	n.n (0.1 µg/kg)	MRL
Streptomycin anddihydrostreptomycin	n.n. (2 µg/kg)	MRL
Sulfonamides and Trimethoprim	n.n. (0.5-2 µg/kg)	MRL

*MRL = forbidden substance (Regulation (EC) No 37/2010/UE);
**n.n. = below the limit of quantification.

Results and discussions regarding the contamination degree of the batch 2

After the analysis of the 15 acacia honey samples, which formed the batch 2, tested in the summer of 2019, the following results were obtained:

The results regarding the **glyphosate residues** determination are below the limit of quantification (0.010 mg/kg), the maximum allowed residue level being 0.050 mg/kg (Regulation (EC) No 369/2005/UE).

Regarding the results of the **nitroimidazole residues** determination, following the used method, LC-MS/MS, there were obtained values below the quantification limit in batch 2, respectively metronidazole < 0.5 µg/kg, dimetridazole < 2.5 µg/kg, ronidazole < 0.5 µg/kg. Considering the limit of quantification, the result was in accordance with Regulation (EC) No 37/2010/UE (regarding the residues of pharmacologically active substances in food products of animal origin).

The **nitrofuran metabolites** determination, bythe LC-MS/MS method showed values below the limit of quantification, respectively (< 1 µg/kg).

Following the **sulfonamides and trimethoprim residues** detection**,** by the LC-MS/MS method, there were obtained results below the quantification limit of the method, respectively below 0.5-2 µg/kg, depending on the analyzed parameter. Considering the limit of quantification, the result was in accordance with Regulation (EC) No 37/2010/UE (regarding the residues of pharmacologically active substances in food products of animal origin).

The **chloramphenicol determination** by the ELISA method, led to results below the quantification limit of the method (< 0.1 µg/kg). Taking into account the quantification limit indicated previously, the result was in accordance with Regulation (EC) No 37/2010/UE (regarding the residues of pharmacologically active substances in food products of animal origin).

The results regarding the **streptomycin and dihydrostreptomycin residues** detection**,** by the LC-MS/MS method, are below the limit of quantification (2 ppb). Taking into account the quantification limit indicated previously, this result was in accordance with Regulation (EC) No 37/2010/UE.

All the results obtained from the analysis of the batch 2 (Table 3) werein accordance with Regulation (EC) No 37/2010/UE.

Table 3. The obtained results regarding the contamination degree of batch 2 of acacia honey

Performed analyses	Result	Allowed limit
Glyphosate	n.n.** (0.010 mg/kg)	0.050 mg/kg
Nitroimidazoles	n.n. (0.5-2.5µg/kg)	MRL*
Nitrofuran metabolites	n.n. (1 µg/kg)	MRL
Chloramphenicol	n.n (0.1 µg/kg)	MRL
Streptomycin anddihydrostreptomycin	n.n. (2µg/kg)	MRL
Sulfonamides andtrimethoprim	n.n. (0.5-2 µg/kg)	MRL

*MRL= forbidden substance (Regulation (EC) No 37/2010/UE);
**n.n. = below the limit of quantification.

Results and discussions regarding the contamination degree of the batch 3

Following the analysis of the 15 polyfloral honey samples, which form the third batch of the present study, the following results were obtained:

Following the **glyphosate residues** analysis by the LC-MS/MS method, the obtained result is

below the limit of quantification (0.010 mg/kg), the maximum residue level allowed being 0.050 mg/kg (Regulation (EC) No 369/2005/UE).

The **chloramphenicol determination** by the ELISA method, led to values (maybe better in English) below the quantification limit of the method (< 0.1µg/kg). Considering the limit of quantification, the result was in accordance with Regulation (EC) No 37/2010/UE.

Following the **nitroimidazole residues** determination (metronidazole, dimetridazole, ronidazole) for the 15 polyfloral honey samples, there were obtained values below the limit of quantification, respectively: metronidazole < 0.5 µg/kg, dimetridazole < 2.5 µg/kg, ronidazole < 0.5 µg/kg. Taking into account the quantification limit indicated previously, the result was in accordance with Regulation (EC) No 37/2010/UE.

The **sulfonamides and trimethoprim residues** detection by the LC-MS/MS method showed values below the quantification limit of the method, respectively below 0.5-2 µg/kg, depending on the analyzed parameter. Considering the limit of quantification, the result was in accordance with Regulation (EC) No 37/2010/UE.

The **macrolide residues** are below the limit of quantification (< 2 µg/kg). Therefore, the result was in accordance with Regulation (EC) No 37/2010/UE.

For the **nitrofuran metabolites** determination (semicarbazide from nitrofurazone, AOZ from furazolidone, AHD from nitrofurantoin, AMOZ from furaltadon), there obtained results were below the limit of quantification, respectively (< 1 µg/kg), the result being in accordance with Regulation (EC) No 37/2010/UE.

Regarding the results of the **tetracycline residues** determinantion for the 15 polyfloral honey samples, the values obtained were below the quantification limit, respectively < 5 µg/kg. Considering the limit of quantification, the result was in accordance with Regulation (EC) No 37/2010/UE.

All the results obtained from the analysis of the batch3 were in accordance with Regulation (EC) No 37/2010/UE.

CONCLUSIONS

The results of toxic substances residues analysis (nitroimidazoles, tetracyclines, macrolides, nitrofuran metabolites, chloramphenicol, streptomycin, dihydrostreptomycin, sulfonamides and trimethoprim**)** for the acacia honey samples, which represent **Batch 1**, are below the limit of quantification.

Regarding Batch 2, consisting of 15 acacia honey samples, the value of glyphosate is < 0.050 mg/kg and the residues of: nitroimidazoles, tetracyclines, macrolides, nitrofuran metabolites, chloramphenicol, streptomycin, dihydrostreptomycin, sulfonamides and trimethoprimare below the limit of quantification.

For the polyfloral honey samples which form the Batch 3, the residues of: nitroimidazoles, tetracyclines, macrolides, nitrofuran metabolites, chloramphenicol, streptomycin, dihydrostreptomycin, sulfonamides and trimethoprim are below the quantification limit.

Following the conducted study on the 3 batches of honey, sourced from local beekeepers and analyzed in the summer of 2019, results were in accordance with the national and international legal requirements.

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Regulation (EC) No 470/2009 of the European Parliament and of the Council laying down Community procedures for the determination of residue limits of pharmacologically active substances in foodstuffs of animal origin.

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