INCIDENCE OF SOME CLASSES OF ANTIBIOTICS IN BEE PRODUCTS. SOURCES OF CONTAMINATION: CASE STUDY ON HONEY AND BEE COLLECTED POLLEN

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Abstract

Bee products are rich in minerals, antioxidants, and simple sugars. Honey is known to be rich in both enzymatic and non-enzymatic antioxidants. Bee-pollen is a complete natural supplement, with high and good protein and aminoacid content, lipids and fatty acids, as well as simple sugars. Beside the basic quality parameters, determination of bee products contamination is of crucial importance. Environmental contaminants in products of the hive involve heavy metals, organic pollutants, pesticides and genetically modified organisms. Also other type of contamination may be due to the improper beekeeping practices. Major contaminants associated with beekeeping practices are acaricides and antibiotics used for the control of bee diseases. Monitoring antibiotics residues in honey and honey products helps to assess the potential risk of these products to human health. APHIS Laboratory have implemented chromatographic methods for determination of different classes of antibiotics. Different samples of honey and bee-pollen were collected from beekeepers and subjected to tetracycline, oxytetracicline and sulfonamide determination. Positive samples were identified, representing 15% from the whole sample numbers.

Key words: antibiotics, beekeeping practices, bee-pollen, chromatography, honey

INTRODUCTION

Bee products are supposed to be natural, man must not interfere in any way in their production, processing, storage and marketing [1]. But specialized laboratories are confronting with the presence of different type of contaminations.

The contamination sources can be divided in apicultural and environmental sources as can be seen in Table 1.

According to European Union regulations, honey and the other bee products, must be free of any contaminants, as they are natural products [2].

If environmental contamination is not solely up to human practices, the apicultural contamination is only the consequence of man practice. Beekeepers use antibiotics for fear of losing their beehives, mainly due to the presence of American Foulbrood Disease, but monitoring bee products in respect of antibiotic presence, is, consumer health protection by a better product quality and not lately the commercial competition [3].

Laboratory for Quality Control of Bee Products from USAMV Cluj-Napoca, have implemented analytical methods for determining the presence of 2 classes of antibiotics: tetracycline (tetracycline and oxytetracycline) and sulfonamides (sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine), using high performance liquid chromatography (HPLC) with photodiode array and fluorescence detection [4, 5]. The importance of monitoring all types of residues in bee products helps the assessing the potential risk of these products to human health and provide data of the incidence in using pesticide treatments on field crops surrounding the hives where honey and pollen are produced.

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Table 1 Type of bee products contamination

A	Apicultural contamination
1	Antibiotics used for bee disease control (chloramphenicol, sulfonamide, streptomycin, tetracycline)
2	Acaricides (synthetic compounds or some non-toxic substances)
3	Paradichlorbenzene (used for wax moth control)
B	**Environmental contamination**
1	Heavy metals
2	Organic pollutants, polychlorinated biphenyls
3	Pesticides (insecticides, fungicides, herbicides)
4	Pathogenic bacteria

MATERIAL AND METHOD

Bee Products Samples

Different type of honeys and fresh bee collected pollen, were screened for the presence of antibiotics during 2018 and 2019 in APHIS Laboratory Cluj-Napoca. Samples were collected directly from beekeepers, from profile fairs and local stores. Honey samples were kept in the dark at room temperature, pollen was kept in the freezer at -18ºC, and the analysis were performed in maximum one week after the collection. Thirty-eight pollen and fifty-three honey samples (black locust, amorpha, linden, rape, multifloral and honeydew declared by the producers), represented the samples for the present screening.

Equipment and Methods

The HPLC system (Shimadzu VP series, Japan), was equipped with binary LC-10AD pump, DGU-14A degasser, SLC-10A system controller, CTO-10AS column oven, SIL-10AF auto-injector and SPD-10A UV-VIS detector set at 360 nm wavelength. The separation of tetracyclynes was performed on a Nucleosil 100 RP-18, 5 µm column, 250x4.6 mm ID with a guard column (4.6 x 7.5 mm ID). For sulfonamide determination the same analytical system was used, but with a fluorescence detector RF-10A XL with excitation wavelength of 405 nm and emission wavelength of 495 nm, and analytical column was a Phenomenex Luna C8 (250 x 4,6 mm, 5µm). As stated before, the validation was made in the lab, registering the retention time of the pure standards, realizing the equation of the concentration curve, determining the limit of detection (LOD) and limit of quantification (LOQ) and also the recovery % [4, 5].

Chemicals and Reagents

Standards of tetracycline and oxytetracycline were purchased from Sigma-Aldrich. Ultra pure water (LC-grade), methanol and acetonitrile (LC grade, Merck KGaA Darmstadt, Germany) and absolute ethanol (reagent grade) were used. The extraction solution was sodium succinate buffer (0.1M succinic acid (Sigma-Aldrich) solution, adjusted to right pH with 5M sodium hydroxide). Also, chelating Sepharose fast Flow resin (GE Healthcare Bio-Sciences Sweden) in 20% ethanol suspension and 10 mM copper sulphate (Chempur, Poland) solution were used for extracting the antibiotics from honey in the sample preparation step. Reference substances of sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine, sulfamerazine and sulfametoxazole were purchased from TitolChimica, Rovigo, Italy. 2M hydrochloric acid, acetonitrile, dichloromethane, 0.1M acetic buffer solution (pH=5), C18 (500mg, 3ml, 45µm) SPE column (Biotage, 0.1M acetic buffer (pH=5), Fluram (Sigma-Aldrich), were used in sample preparation for the sulfonamide determination.

Statistical Analysis

All determinations were made in duplicate. Fortified samples were made, when positive results were obtained, for result confirmation.

RESULTS AND DISCUSSIONS

The major bee diseases for which antibiotics are applied are American and European Foulbrood, caused by different bacteries and Nosema disease, caused by a microsporidian. European Union do not

allow any veterinary medicinal product containing antibiotics in beekeeping, and bee products. Anyway at European level, a technical guide has been published by the Community Reference Laboratories [6]. The purpose of this technical guide is improveing and harmonising the performance of analytical methods for substances for which MRLs have not been set. In this guide, recommended concentrations (RCs) in honey has been given for the tetracyclines (20 ppb), sulfonamides (50 ppb), streptomycin (40 ppb) and macrolides (tylosin and erythromycin, 20 ppb). These RCs, however, have no real legal basis. Antibiotics are found in honey because are used in apiculture practice for treatment of bacterial diseases (in higher concentrations), or as "growth promotors" (in lower concentrations). This is, however, an improper beekeeping practice, because antibiotics are forbidden to be used in the hive, due to their remanence in the bee-products [7]. The presence of antibiotics in bee products may have direct toxic effects for consumers [8], and their monitoring helps to assess the risk to human health and developing antibiotic resistance.

Contamination Level of Honey

Sulfonamine Contamination

It is known that the level of sulfonamides (sulfanilamide, sulfacetamide, sulfadiazine, sulfathiazole, sulfamethazine, sulphametazine, sulfametoxazole) in honey decreases over time, if the sample is kept at room temperature [9]. But the reduction is only apparent, because glucose adducts are formed, and the antibiotic remains in the composition, but is bounded to sugars. For this reason, in the sample preparation one of the steps is hydrolysis (acidic), to ensure the complete release of bounded residues from the matrix.

Calibration curves of the standards, were performed for each sulfonamide in the range of 3-75 µg/kg. Correlation coefficient values were higher than 0.995. All compounds were in baseline separation, with a good resolution (Fig.1). Limits of detection were calculated using the HPLC soft. The chromatographic peaks in the samples were identified by comparing the retention data obtained for the standards and the spiked sample with the standards under same conditions and using the fluorescence detector to measure the spectrum while the mobile phase pass through the chromatographic column.

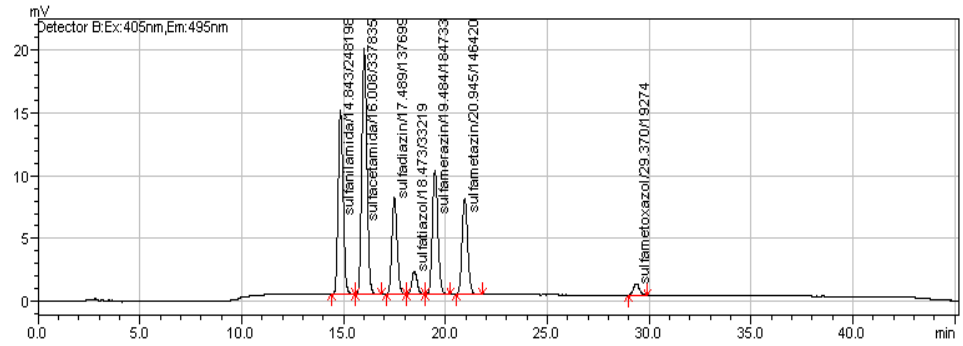


Fig. 1 HPLC-FL chromatogram of seven sulfonamide standards

Fifty-three samples of honey were screened for the presence of sulfonamides. No residues from this class of antibiotics were found present in the honey samples collected for this experiment. The method used in our laboratory, as well as the separation of the seven sulfonamides, was in accordance with the study of Posyniak et al. [10].

Tetracycline Contamination

In the last two years, many studies were made in our laboratory for determining the incidence of antibiotic contamination in bee products, namely honey and bee collected pollen. The same samples of honey were

screened for the presence of tetracyclines (tetracycline and oxytetracycline) and 15.09% of them were found positive for oxytetracycline. No tetracycline residues were found in honey samples. Whenever is necessary, fortification of the samples which present suspicions or when checking the accuracy of the method, chromatograms are registered (Fig. 2).

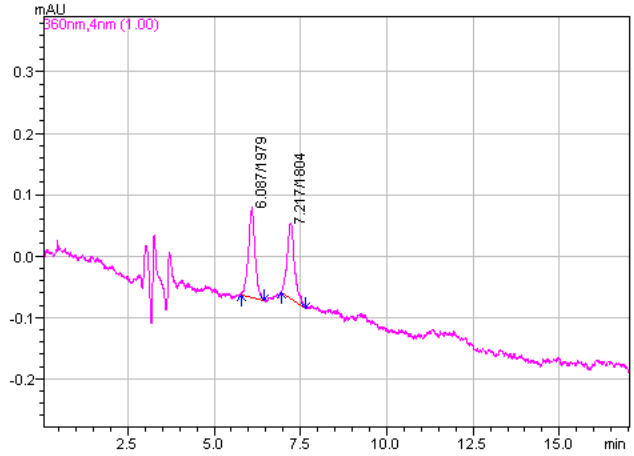


Fig. 2 HPLC-PDA chromatogram of tetracycline and oxytetracycline standards

From the 53 tested honey samples, 8 were found positive for oxytetracycline, and no tetracycline was detected in honey. The contamination level lies between 1.76 ppb and 13.1 ppb. The amount of oxytetracycline is shown in Table 2.

Table 2 Contamination level of honeys found positive for tetracyclines

Sample code	Tetracycline content (ppb)	Oxytetracycline content (ppb)
H5	-	12.99±0.01
H6	-	13.1±0.00
H8	-	1.76±0.02
H12	-	8.56±0.01
H23	-	5.82±0.01
H27	-	9.73±0.02
H28	-	8.82±0.01

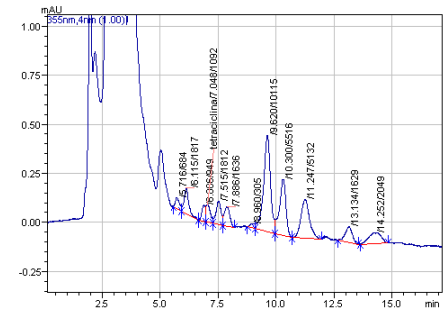
Contamination Level of Beepollen

Thirty-eight samples of beepollen (35 fresh pollen samples and 3 dried pollen samples), were collected from beekeepers and acquirers to be screened for the presence of tetracycline. In 2018, one bee products acquirer in our area, notify our laboratory to verify a pollen sample (mixture from several beekeepers), which was found positive for tetracyclines by another laboratory. The sample presented a high concentration of oxytetracycline (66.74 ppb), and at our request, the acquirer provide us all the samples that consist the batch pollen mixture, separately. Analyzing again the samples (4 distinct samples of bee pollen), three of them were found negative and one was positive, with an extremely high concentration of oxytetracycline (280.0 ppb)(Table 3). At the end of the study, from the 38 analyzed samples of bee pollen, 5 were found positive for oxytetracycline and one for tetracycline, representing 15.79% positive results from the analyzed samples.

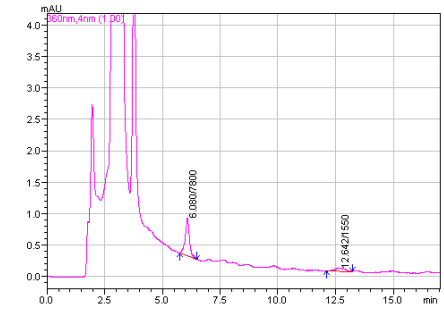
Table 3 Contamination level of bee-pollen found positive for tetracyclines

Sample code	Tetracycline content (ppb)	Oxytetracycline content (ppb)
P1	-	66.74±0.21
P5	-	280.0±0.31
P8	-	11.82±0.08
P16	-	290.59±0.84
P18	19.4±0.01	-
P32	-	53.49±0.23

Fig. 3 presents the chromatograms registered for two samples of bee pollen found positive for tetracycline (19.4 ppb)(A) and for oxytetracycline (66.74 ppb)(B).



A



B

Fig. 3 Chromatograms registered for sample P18, found positive for tetracycline (A), and sample P1, found positive for oxytetracycline (B)

If the presence of antibiotics in honey is somehow logic (beekeeper treatment against different bee diseases, especially Fouldrood), finding tetracyclines in bee-pollen is at first sight questionable: where can be the source of this contamination?

Multiple answers were given by the beekeepers, but scientifically there is no study to confirm the theories. One study made in France [11] show the persistence of tetracycline residues in honey after treatments, indicating the persistence in the hive of the antibiotic, but also the diffusion into the apiary. This might be one of the answers for the presence on antibiotics in bee-pollen. If the bees take the antibiotic in their bodies, outside the hive, it may be mixed with flower pollen when combining with their own substances, in the formation of the pollen grain. Other explanation may be the presence in the apiary vicinity of birds or animal farms with open air feeding with concentrated fodder and antibiotic substances. But the most credible way in which the antibiotic reached bee-pollen is that the bees collect pollen from plants that were treated with different insecticides that have in their composition these types of contaminants, if not in free form, than as metabolites belonging to different chemical classes. Such a study was made in Switzerland [12], confirming this hypothesis.

CONCLUSION

Although antibiotics are banned in apiculture, there still are beekeepers that use tetracycline and/or oxytetracycline in bee colony management.

Regardless the way of getting into bee products, these classes of contaminants are banned, and their presence makes the product un-usable for commercialization and human consumption. Moreover, World Health Organization has recommended that antibiotics that are licensed in human medicine should be forbidden in treatment or as growth promoters in livestock [8]. Antibiotic residues cannot be eliminated completely from bee products, regardless of time, production and harvest techniques or storage. For this reason, proper monitoring is needed, by complying with quality assurance, certification and legislation.

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