Honey producers and consumers

Tetracyclines (TCs), a family of antibiotics with broad-spectrum activity, are frequently used to treat bacterial infections. Its use as veterinary drug is banned in the EU, but is still widely used in countries like USA, Canada, Australia, India, Argentina [1-3].

Honey is being used as a pure natural and as an ingredient in many foods, pharmaceuticals and cosmetics than ever before, so honey testing has become essential to maintain its healthful characteristics and protect public health. TCs are used for the treatment or prevention of American and European foul broad in bee colonies which are caused especially by two species of bacteria - Paenibacillus larvae and Melissococcus pluton [4].

Honey is defined as the natural sweet substance produced by Apis mellifera bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature [5]. Through the content of its composites bearing antioxidant effect, the honey could contribute to decreasing/preventing the oxidative stress [6].

Antibiotics can accumulate in bee hives and migrate from the hives to honey, propolis, royal jelly and wax, resulting in contamination of these bee products [7].

Honey and bee products have the image of being natural, healthy and clean [8]. The presence of TC and its degradation products in honey may have harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth and gastro-intestinal disturbance due to the selective pressure of antibiotics on the micro flora of human gut [9].

Muhammad et al. (2009) reported that indirect and long term consequence of the ingestion of low-dose of antibiotics by consumers include microbiological effects, carcinogenicity, reproductive effects and teratogenicity [10].

The consumer is often faced with worthless substitutes but sometimes also with a dangerous cocktail of chemicals such as antibiotics, colourings and hydroxymethyl furfural (HMF) in honey [11].

Good agricultural practices, good beekeeping practices, good hygiene practices, and good manufacturing practices all apply to honey production. The use of these good practices from the supply of inputs through to product distribution promotes quality during production, processing, and packaging and provides quality assurance and accreditation to verify honey quality. Some countries, such as Australia, Canada, New Zealand, USA and Japan, have adopted national best practice guidelines for the production and distribution of honey [12].

Tetracyclines group

The history of TCs involves the collective contributions of thousands of dedicated researchers, scientists, clinicians, and business executives over the course of more than 60 years [13].

TCs produced by Streptomyces spp. are broad-spectrum agents, exhibiting activity against wide range of Gram-positive and Gram-negative bacteria, parasites, atypical bacteria (chlamydiae, rickettsiae, mycoplasmas). The members of the TCs group include tetracycline (TC), chlortetracycline (CTC), oxytetracycline (OTC) and doxycycline (DC) [14].

TCs are broad-spectrum antibiotics that consist of a substituted 2-napthacenecarboxamide molecule. They are widely used in veterinary medicine for cost-effective prophylactic and therapeutic treatment. TC antibiotics are protein synthesis inhibitors, inhibiting the binding of aminoacyl-RNA to the mRNA-ribosome complex [15]. The structures of TCs are presented in figure 1.

Figure 2 shows TCs 3D spectra in honey, in which optimal resolution can be observed [17].

Epi-tetracycline (ETC), epi-anhydrotetracycline (EATC) and anhydrotetracycline (ATC) (fig. 3) may be present in TC as impurities. These compounds may form during storage under adverse conditions of temperature and humidity. Anhydro derivatives may also be found in out-of-date samples of TC. These compounds are either inactive as antibiotics or toxic. Hence, the permitted concentrations of these impurities in pharmaceutical preparations fixed by the European Pharmacopoeia are 0.5% ETC and 0.05% EATC and ATC [18].

ETC is the major degradation product of TC in honey [19]. Therefore, it was not surprising that TC was found mostly together with ETC. OTC was also found several times though it is known to be chemically unstable in honey [20].
A challenge in TC 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 TC and its epimer, that is, TC and ETC, OTC and epi-oxytetracycline (EOTC), CTC and epi-chlortetracycline (ECTC) [21].

Legislation of honey
In order to guarantee the nomination of honey and also protect human health, the use of antimicrobials in apiculture is usually strictly regulated or banned. According to Regulation (EC) No 470/2009 [21] and Regulation (EU) No 37/2010 [22], in the European Union, no maximum residue level (MRL) for TC and any other antibacterial substance residues in honey are allowed.

Despite this decision, some countries have established action limits or tolerated levels for TC in honey. For instance, in Belgium, the action limit for the group of TC has been fixed at 20 μg/kg. France applies a nonconformity limit for TC in honey of 15 μg/kg, the reporting limit in Great Britain is 50 μg/kg, while the tolerance level in Switzerland is 20 μg/kg. In Japan, based on microbiological research, a value of 0.1 mg/kg was introduced as the allowed residual quantity of TC in honey. Australia, Indian, American and the US Food and Drug Administration (USFDA) have set MRL for only OTC in honey at 300 ppb [4]. Worldwide limits for TCs in honey can be observed in table 1.

Methods for the determination of TCs residues in honey
In routine honey analysis TCs are generally tested by:
1. screening, determination of positive samples: Charm II Test, ELISA

| Country/Regulation                        | Maximum residue limits (μg/kg) | References |
|-------------------------------------------|-------------------------------|------------|
| **Codex Alimentarius**                    | TC   | CTC  | OTC  | DC  | [23]             |
| EU, European Regulation 37/2010           | -    | -    | -    | -   | [23]             |
| USA, Code of Federal Regulations - Title 21 Part 556 | -    | -    | 300  | -   | [24]             |
| Canada, List of Maximum Residue Limits for Veterinary Drugs in Foods | -    | -    | 300  | -   | [23]             |
| Australia/New Zealand, Food Standards 14.2 - Schedule 20 | -    | -    | 300  | -   | [25]             |
| Brazil, Normative Instruction 11/8.3.2017 | 20   | 20   | 20   | 20  | [27]             |
| Japan, The Japanese Positive List System for Chemical Residues in Foods | 0.1  | -    | -    | -   | [28]             |
| India - FIC, Standards for Honey and prohibition of antibiotics | 5    | 5    | -    | -   | [29]             |
2. Quantitative determination of positive samples by HPLC, LC-MS [8, 30-32]. Different analytical methods are reported for the determination of TC residues in honey. These include HPLC with UV detection [33-40], HPLC with fluorescence detection [41, 42], liquid chromatography with mass spectrometry [43, 44]. Nowadays, liquid chromatography coupled with mass spectrometry and tandem mass spectrometry seem to be the techniques of choice for analysis of these groups of antibiotics [45].

For the first time Wanga et al., (2018) report a tetrahedron-assisted aptamer-based SPR (Surface Plasmon Resonance) biosensor for automatic screening of small molecules. The aptasensor was then validated in real-world application for tetracycline screening in multiple honey samples, achieving good recovery rates of 80.20-114.3%, intuitive sensorgrams indicating the binding kinetic properties, and high specificity towards tetracycline. Figure 4 illustrates the construction of SPR aptasensor [46].

Because of their polar nature, TCs have the ability to strongly bind to proteins as well as to chelate with divalent metal ions. The analytical steps of each selected method (sample treatment, analytical technique, and detection limits) are summarized in tables 2 [47].

International reports of TCs residues in honey samples

There are several international reports of antibiotic residues in honey samples. A total of 567 Basque honey samples were analyzed with the Charm II system. 24 samples were presumptive positive for TCs [56]. The residues were confirmed by liquid chromatography fluorescence detection (LC-FD) and tandem mass spectrometry (LC-MS/MS), according to the latest EU criteria for the analyses of veterinary drug residues [57]. The TC levels was from 15 to 920 µg/kg. Residues of veterinary drugs were confirmed in a very limited number of honey samples: tetracycline (4.22%) [56]. In another Spanish study by Vidal et al. (2009), in which 251 honey samples was analyzed. 19% of the samples have found to be contaminated by the residue of TC [58].

In a study in which 251 honey samples collected across Greece were analyzed by LC to detect TC - derived residues, 29% of the samples had TC residues. The range of the detected amounts of each observed drug residue in the examined samples was 0.018-0.057 µg/kg, 0.023-0.335 µg/kg, 0.018-0.190 µg/kg and 0.013-0.393 µg/kg for TC, OTC, DC and CTC respectively. The reason for this frequent use could have arisen from easier access connected with pricing, flexibility of use or the need to go above the normal dose in response to dwindling efficacy [59].

| Compounds | Extraction/ clean-up | Separation | Equipment | LOD (µg/kg) | References |
|-----------|---------------------|------------|-----------|-------------|------------|
| CTC, DC, MINO, TC, MTC, OTC | MacDonlane buffer (Na2EDTA, pH 4.0/phenyl-SPE) | Discovery RP-Amide C18 (3.0 µm) | Gradient: 0.09% OA (pH 3.0)/CAN | LC-DAD | 15.30 | [44] |
| CTC, DC, OTC, TC | 50 mM oxalic buffer (pH 4.0)/Oasis HLB-SPE | Atlantis dC18 (150 x 2.1 mm, 3 µm) | Gradient: 1% FA/ACN:MeOH (50:50, v/v) | LC-MS/MS | 3.3 | [48] |
| CTC, OTC, TC | MacDonlane buffer (Na2EDTA, pH 4.0)/hexane, PLS-2-SPE* | Hydrophobic C18 HS:SO1-3 (100 x 4.6 mm, 3.0 µm) | Isocratic: 1% imidazole buffer/MeOH (82:18, v/v) | LC-FLD | 5.9 | [49] |
| CTC, OTC, TC | Citrate buffer, (Na2EDTA)/PLS-2-SPE* | TSK-gel ODS-80Ts (150 x 4.6 mm) | Isocratic: 1% imidazole buffer/MeOH (75:25, v/v) | LC-DAD | 10-20 | [50] |
| CTC, DC, OTC, TC | 5% HCl/MIP-SPE | Restek C18 (150 x 2.1 mm, 5.0 µm) | Isocratic: 100 mM OA/ACN/MeOH (70:20:10, v/v/v) | LC-MS/MS | 0.1-0.3 | [51] |
| CTC, OTC, TC | ACN/SPE (home-made sorbent) | ShodeX-RSpak DE-613 (150 x 6.0 mm) | Isocratic: 0.05% TFA/ACN (50:40, v/v) | LC-MS/MS | 3-20 | [52] |
| CTC, OTC, TC | 50 mM NaOH/ac buffer (pH 5.5)/MCAC-SPE, Oasis HLB-SPE | Waters Phenyl (100 x 2.1 mm, 3.5 µm) | Gradient: 0.1% FA/0.1% FA in ACN:MeOH (50:50, v/v) | LC-MS/MS | 1.2-7.7 | [53] |
| CTC, TC | MacDonlane Buffer (pH 4.0)/Strata X.SPE | Symmetry C18 (150 x 2.1 mm, 3.5 µm) | Gradient: 0.05% AcOH/0.05% AcOH in CAN | LC-MS/MS | 5.5-9.2 | [54] |
| OTC, TC | Water/chitosan modified graphitized | Se-C18 (50 x 4.6 mm, 5 µm) | Gradient: 0.1% FA/MeOH | LC-HRMS | 0.5-10 | [55] |

*DMC - demecycline; MINO - minocycline; MTC - methacycline.
PLS-2, polyethylene-divinylbenzene polymer (RP-SPE).
*LOD - limit of detection.
Reybroeck (2003) monitored 248 samples of locally produced and imported honey on the Belgian market for the presence of residues of antibiotics in the period 2000-2001. According to them residues of antibiotics were found in a very limited number of honey samples produced in Belgium and TC was detected in 2 out of 72, samples [31].

In China the near infrared spectrum detection technology (NIR) has been used in the detection of TCs residues in 153 honey samples. The TC content in honey was very low (10^-7 - 10^-9), in 41 samples [60].

50 honey samples collected from Southern Marmara region in Turkey were analyzed for the presence of OTC residues by using LC-MS system. Samples were free from residues [61].

30 German and 47 imported non-European honeys were analyzed for TCs. 22 of the imported honeys contained residues (in most cases more than one), whereas 29 of the 30 German samples were free of residues [62].

A total of 16 samples of honey were collected from Ethiopia. TC analysis was done using a Tetrasensor test. No TC residues were detected [63].

In France, TC residues were detected in honey after a treatment in hives, indicating their persistence and diffusion into the apiary. These results showed that the TC must be used with precaution in honey production [64].

In UK, a study aimed to assess OTC residue levels in honey after treatment of honeybee colonies with two methods of application in liquid sucrose and in powdered icing sugar. The samples of honey were extracted up to 12 weeks after treatment. It was demonstrated that the method of application of OTC in liquid form results in high residue levels in honey with residues of 3.7 mg/kg, eight weeks after application [65].

The results obtained in antibiotic analysis of 130 samples shows that honey have no content of TCs. This shows that Romanian polyflora honey is superior than others, the most valuable honey is harvested from Transylvania region, an area of the mountain, which raises the value and prestige of Romanian honey [66].

Table 3 summarizes the analytical performance for the detection of tetracyclines reported in the literature.

Residual levels of contaminants cannot be changed through various production techniques; therefore, adequate monitoring is required [73, 74].

**Conclusions**

Honey is a natural product that is widely used for both nutritional and medicinal purposes. TCs consumed along with honey can produce resistance among bacteria in the consumers and consequently, there is difficulty in treating many infections in humans. There are studies [75,76] that demonstrate bacterial resistance to antibiotics as a result of their abusive or coincidental use such as the use of honey with significant content in antibiotics such as tetracycline.

It is useful, under specific circumstances, that new substances - perhaps plant derived products of limited adverse reaction potential - and non-aggressive alternative treatments to be investigated and used [77-80] for the prevention and management of chronic diseases.

TCs residues in honey originate mostly from improper beekeeping practices and not from the environment. Beekeepers should aware and reduce the use of antibiotics in bee honey in order to control honey quality and for the safety of consumers.

Standardized and updateable analytical protocols need to be established to determine contamination of honey.

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