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OXYTETRACYCLINE AND SULPHONAMIDE RESIDUES ANALYSIS OF HONEY SAMPLES FROM SOUTHERN MARMARA REGION IN TURKEY

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Abstract

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Honey samples collected from Southern Marmara region in Turkey were analyzed for the presence of oxytetracycline and sulphonamides (sulfadiazine, sulfathiazole, sulfamerazine, sulfadimethoxine) residues. A total of 50 samples composed from multi-flavour, chestnut, pine and linden honeys that were collected from hives at different locations in the region were monitored by using LC-MS system. Tested residues were not detected in any of the tested hives indicating that either their concentrations were lower for being detected by the used system or samples were free from residues.

Key words: honey, oxytetracycline, sulfonamides, residue, antibiotics, LC-ESI-MS
Abbreviations: Maximum residue limits (MRLs); American foulbrood (AFB); European foulbrood (EFB); Liquid chromatography; Electrospray ionization mass spectrometry LC-ESI-MS; Oxytetracycline (OCT); Selected reaction monitoring (SRM)

Introduction

Honey is a natural and healthy product consumed around the world. However, in apiculture some beekeepers use antibiotics against the bacterial diseases known as American and European foulbrood (AFB and EFB) highly contagious and destructive diseases that affects honeybees, caused by *Paenibacillus larvae* and *Melissococcus pluton* (*Bacillus larvae*) respectively (Reybroeck, 2003). As a result, antibiotics can be detected at trace levels in honey of treated bees. Tetracyclines and sulfonamides are broad-spectrum antibiotics commonly used all over the world in human and veterinary medicine for therapeutic and prophylactic purposes against most Gram-positive and many Gram-negative microorganisms (Carrasco-Pancorbo et al., 2008; Oka et al., 1997; Oka et al., 1998;). These substances are also used as feed addi-

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tives in animal husbandry. Their residues were found in several animal products such as honey, milk, eggs, fish or meat (Khosrokhavar et al., 2008; Hassan et al., 2007; Coyne et al., 2004; Bing et al., 2005).

In 1990, the Commission of the European Union laid down the procedure for establishing maximum residue limits (MRLs) of veterinary drugs in foodstuffs of animal origin (EEC-Regulation 2377/90 and amendments). However, no MRLs have been fixed for bee products (Hammel et al., 2008). Some countries like Belgium and The United Kingdom have defined their own limits at 20 and 50 g/kg, respectively for the sum of all sulfonamides. The limit established by Switzerland is 50 g/kg (Mohamed et al., 2007). This is also the case for tetracyclines. While some countries did not fix any MRL for tetracyclines in honey, some others fixed some limits in order to make the situation clearer. In Switzerland and Belgium, the limit was set at 20 g/kg while in Great Britain and France was at 50 and 15 g/kg respectively (Li et al., 2008). In Turkey the use of antibiotics in apiculture had been banned by legal authorities (Gunes et al., 2008).

Antibiotic residues show a relatively long half-life and they may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals and disorder of the haemopoietic system, or cause problems indirectly through induction of resistant strains of bacteria (Horie et al., 1998; Dubois et al., 2001; Verzegnassi et al., 2002; Huebra et al., 2004; Tillotson et al., 2006). Therefore, the presence and MRLs values of antibiotic residues in honey should be regulated.

Microbiological tests are widely used to analyze residues in honey but these tests are qualitative and not very sensitive. New analytical techniques such as LC-ESI-MS and LC-ESI-MS/MS methods have been developed and exhibited good accuracy and ruggedness (Benetti et al., 2006; Wang, 2004). In the present study, we used LC-ESI-MS technique to analyze honey samples collected from hives the Southern Marmara region for residual oxytetracycline and sulphonamides.

Materials and Methods

Honey samples

Between January 2004 and August 2005, 50 honey samples composed from multi-flavor, chestnut, pine and linden honeys were collected from different hives located in different area in Southern Marmara region. Samples were taken carefully from hives, thus mixing of honeys originating from different hives was not allowed. All samples were stored at room temperature in a dark and dry place until laboratory analysis.

Chemicals and apparatus

All chemicals and solvents used were HPLC grade. Standard oxytetracycline and sulphonamide antibiotics (Sigma, St. Louis MO, USA) were obtained from Riedel-de-Haen (Seelze, Germany). LC-MS measurements were carried out with Agilant 1100 Series chromatography coupled to LC/MSD Trap SL mass spectrometer with a C18 column (Zorbax, 3.5 µm, 2.1x30 mm I.D.) and ESI technique.

Extraction and clean-up procedures

Oxytetracycline (OCT) extraction

Extraction and measurement of OCT was performed according to Vinas et al. (2004) with some modifications. Briefly, 3 g honey was dissolved in 6 ml of 0.1 M Na_2EDTA-Mcllvaine buffer (PH 4.0), vortexes thoroughly, centrifuged at 800 rpm for 10 min and applied to a Bont Elut ENV solid-phase extraction cartridge (Harbor City, USA) that was pretreated with methanol (6ml) and water (6 ml), and air-dried by aspiration for 5 min. OCT residue was eluted from the column with 10 ml of methanol. The elute was first evaporated to dryness under reduced pressure at 30°C and then dissolved in 1 ml of water-methanol (50:50,v/v) combination. 10 µl of elute was injected into the LC-MS system.

Standard OCT solution was prepared at a concentration of 1 mg/ml in distilled water and kept at –20°C. The solution was diluted to the required concentrations with distilled water before use.

Sulphonamides extraction

For extracting procedure, the modified method of

Verzegnassi et al. (2002) was used. Briefly, 5 g sample was dissolved in 5 ml of tricloreacetic acid (10 % v/v) by agitating on a mechanical shaker for 10 min and heated to 63 ± 2°C for 60 min in a water bath. Then, the sample was cooled to the room temperature and the pH was adjusted to 6.5 by using Na_2HPO_4 (1 M, pH 12). 10 ml of acetonitrile and 2.5 ml of dichloromethane were added and the mixture was vortexed for 10 min. The mixture was then centrifuged for 15 min at 5000 g (Sigma, Germany) at 10^0C. The superior part was taken in clean tube and the extraction was repeated. Thereafter, the combined mixture was evaporated to dryness (Thermo Savant SPD SpeedVac, USA). The residue was taken up in 1ml of water-acetonitrile (90:10 v/v) and an aliquot of 10 µl was injected onto the LC-MS system.

Standard sulphonamide solutions were prepared at a concentration of 1 mg/ml in methanol and kept at – 20^{o}C. Working standards were prepared daily by diluting stock solutions with distilled water.

LC-ESI-MS and Calibration curves

LC-MS measurements were carried out with Agilant 1100 Series chromatography coupled to LC/MSD Trap SL mass spectrometer. A linear gradient from 100% solvent A (0.3% formic acid and 5% acetonitrile in water, v/v/v) at 0 min to 70% solvent A and 30% solvent B (0.3% formic acid in pure acetonitrile, v/v) at 14 min was applied. At 14 min, solvent A was increased to 100% until 20 min at a flow-rate of 0.2 ml/min. The LC column and autosampler temperatures were set at 35 and 5°C respectively. The analytes were detected using electrospraying the positive ionization mode. The nebuliser gas flow was set to 80 l/h and the desolvation gas flow to 600-650 l/h. Selected reaction monitoring (SRM) was used for identification of proper compound.

For quantification of oxytetracycline and sulphanamides in the honey samples, calibration curves using different blank honeys were constructed. Honey samples were fortified in standard solutions to have a final concentration about 6, 12, 30, 60, 150, 300 ng g^{-1}. All samples were injected in triplicate. Area ratios of the SRM transition showing the most intense signal and standard were plotted against their respective amount ratios.

Results and Discussion

The Southern Marmara is an important region for honey production in Turkey. Nearly 20% of the total honey produced is originated from this region. For a long time oxytetracycline and sulphonamides were used to protect honey bees from infections. But systematic use of these antibiotics conferred resistance. In Turkey, during the last decade due to the raised resistance, usage of these antibiotics have been banned by legal authorities, thus only the use of erythromycin containing products were allowed. But, thereafter because of the health problems and the requirements of EU the usage of all antibiotics in beekeeping was prohibited by legal authorities.

The LC-ESI-MS method was tested for tR value, base peak for protonated molecule (m/z), limit of detection (LOD) value, repeatability, intermediate re-

Table 1

Calibration characteristics of the technique

Antibiotics	t_R, min	Precursor (M+H) ion m/z	LOD, ng g^{-1}	Repeatability %, n=10	Intermediate reproducibility %, n=10	% Recovery , (n=5)		
						10	50	100
						(ng g^{-1})		
Oxytetracycline	7.8	461	10	1.5	3	91	89	92
Sulfadiazine	2.2	251	12	2	2.9	80	80	82
Sulfathiasole	3.1	256	6	1.8	2.5	85	87	85
Sulfamerazine	3.5	265	11	2	2.7	80	80	81
Sulfadimethoxine	12.8	311	9	1.5	2.2	85	86	85

producibility and % recovery for each of tested antibiotic residues spiked in honey matrix to determine its accuracy and precision, and the results are shown in Table 1. Limit of detection (LOD) value varied between 6 -12 ng g^{-1} depending upon the antibiotic. The recovery analysis was tested by using three different concentrations of 10, 50 and 100 ng g^{-1} of each residue and highest yield was obtained with oxytetracycline giving 89-92% recovery. By using these parameters 50 honey samples were analyzed and any of the samples were found to be contaminated with tested residues indicating that either their concentrations were lower for being detected by the used system or samples were free from residues or even they were used, over the time they were degraded in honey matrix.

It should be kept in mind that residue level may be decreased in honey matrix over the time. Dinkov et al. (2005) compared the elimination half-lives of tetracycline and oxytetracycline in a feeding assay conducted by adding antibiotics in sugar solution. Authors reported that, over 126 day's experimental period, elimination of tetracycline was 4 times greater compared to oxytetracycline. Another survey conducted on the oxytetracycline spiked multifloral samples revealed that majority of the antibiotic was eliminated after 120 days (Vangelov and Parvanov, 1992). Similar observations were also reported for sulphonamides residues when honeys were stored at room temperature. The reduction in the level was attributed to the formation of glucose adducts (N4 –glucopyranosyl derivatives) (Schwaiger and Schuch, 2000).

Conclusion

The employed method showed good accuracy for determination of oxytetracycline and sulphonamide residues from honey. The usage of antibiotics in apiculture should be controlled and consciousness of beekeepers should be improved by organizing informative forums.

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