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A survey of sulfonamides in meat and honey in Vojvodina Market, Serbia

Sulfonamide in Fleisch und Honig auf einem Markt in Vojvodina, Serbien – eine Übersicht

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Summary

Sulfonamides are a class of antimicrobials used for the treatment of food producing animals. Administration of sulfonamides is governed by relevant legal framework and they are prohibited from use in certain animal species (e.g. honeybees, laying hens). The objective of this study was to compare the presence and contents of sulfonamide residues in products originating from the animals that are not allowed to be administered sulfonamide and the animals for which sulfonamides are used as allowed substance. A total of 295 samples (180 honey samples and 115 meat samples) were examined using HPLC with fluorescence detector (HPLC/FLD). The presence of residues was detected in seven (6.1 %) meat samples. The amount of sulfonamide residues was less than 100 µg/kg, which is considered permissible residue level. The prohibited sulfonamide residues were found in six (3.3 %) honey samples. Unauthorized administration of veterinary drugs is obvious and can result in a high level of residues in foodstuffs and high risk for human health. Out of six honey samples with residues, even 42.8 % had sulfonamide levels higher than 100 µg/kg. Compliance with guidelines for good production practice in primary production ensures safety of foodstuffs on the market. Prevention of illegal administration of veterinary drugs is particularly difficult. However, relevant measures should be taken to minimize it through adequate education of farmers and animal breeders about the threat they pose for consumers and community.

Keywords: sulfonamides, residue, illegal use, honey, meat

Introduction

Sulfonamides are synthetic, antimicrobial drugs of broad spectrum inhibiting both gram-positive and gram-negative bacteria, even some protozoa *(Toxoplasma, Plasmodium, Coccidia)*. They are one of the most used antimicrobials in veterinary medicine and are approved for the treatment of urinary, digestive and respiratory infections in animals (Boxall et al., 2003; Cháfer-Pericás et al., 2010).

Therapeutic use of sulfonamides in food producing animals inevitably leads to the residues in edible tissues. The safety of foodstuffs originating from treated animals is ensured by complying with the instructions for drug use and relevant withdrawal periods. Misuse of veterinary drugs (inappropriate dose, reduced or prolonged application period) or illegal drug use for animal species whose treatment is prohibited can lead to uncontrolled presence of residues, mostly at levels that have harmful effects on consumers' health. Adverse effects of sulfonamides in foodstuffs predominantly include potential allergic reactions in consumers, transmission of bacterial resistance gene as well as imbalance of physiological microflora. Carcinogenic and teratogenic effects have been reported in cases of prolonged administration of high therapeutic doses of sulfonamides (Littlefield et al., 1990; Andrew et al., 2001).

Honey is a foodstuff of high biological value, commonly used by healthy population. It is highly popular among immunocompromised persons because of its natural antimicrobial properties. Safety standards set for honey are very strict (Wei et al., 2012). The residues of antimicrobial drugs are a significant chemical hazard related to the safety of honey. Sulfonamide residues in honey detected after the treatment of a honeybee brood in the USA affected by American Foulbrood have led to the prohibition of sulfonamides in the therapy of honeybee diseases (Barganska et al., 2011). Sulfonamide residues in honey appear as a result of their intentional and illegal administration for the prevention and therapy of honeybee diseases. High risk from residues in honey is the reason why antimicrobial drugs usage in Serbia and the majority of EU countries is prohibited. MRL values for total sulfonamides enacted by national legislation in some countries, range from 20 µg/kg (Belgium), 50 µg/kg (Switzerland and the UK) to even 100 µg/kg in Brazil (Granja et al, 2008; EC, 2010; Zhan et al., 2019).

Sulfonamides are approved for therapy of poultry, pigs and cattle but not for all categories. They are prohibited for therapy of laying hens and dairy cows. Appropriate drug use, in accordance with producer's labeling and withdrawal ensures that meat does not contain any harmful drug residues (above MRLs). MRL value for total sulfonamide in milk, meat, liver, kidneys and adipose tissue is 100 µg/kg, according to Commission Regulations (EU) No 2377/1990 and 37/2010.

Residue monitoring implemented in the EU during 2016 shows that the levels for group B1 (antibacterial substances including sulfonamides) are above MRL in cattle (0.4–0.8 % of samples), swine (0.1–2.0 % of samples) and honey (2–10 % of samples). Thus, honey is considered the first most risky foodstuff in the EU market. Most commonly detected sulfonamide residues include sulfadiazine, sulfadimethoxine and sulfamethazine (EFSA, 2018). The analysis of honey available on the Serbian market for the presence of residues is highly justified having in mind that the hazards that are well-established in the EU and suspected illegal administration of antimicrobials in apiculture in Serbia.

The risks associated with veterinary drug residues in food are controlled during primary production. Thus, the aim of this study was to compare the residues in primary production of animals in cases when the use of sulfonamides is permitted with the production when sulfonamides are prohibited. The study will also examine potential health threats for consumers, the presence of unsafe foodstuffs in the market – meat and honey with increased sulfonamide levels (above MRL values) and dietary exposure assessment.

Material and methods

Samples

Honey samples amounting 500–750 g were collected randomly from supermarkets and open markets in Vojvodina during the period from August to October 2018. A total of 180 honey samples produced in Vojvodina were examined: 78 meadow, 59 acacia, 20 linden, 10 sunflower, 7 flower samples and 6 samples of forest honey. Before the analysis, all honey samples were stored in glass jars, in the dark, at room temperature.

Meat samples weighing 300–500 g were collected randomly from supermarkets in Vojvodina during the period from January 2018 to January 2019. A total of 115 meat samples were examined including 83 pork, 19 beef and 13 poultry samples.

Reagents and Chemicals

All chemicals and reagents used were of analytical grade with high purity. Acetonitrile, methanol, acetone, fluorescamine, HCl, citrate buffer, NaOH and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anhydrous magnesium sulphate (MgSO4), anhydrous sodium acetate (CH3COONa), primary and secondary amine (PSA), and octadecyl (C18) used were obtained from Merck (Darmstadt, Germany). Standard solutions were prepared using 10 sulfonamides from Dr. Ehrenstorfer GmbH, Germany: Sulfachlorpyridazine (Lot. 107253), Sulfamethazine (Lot. 91440), Sulfamerazine (Lot. 92691), Sulfamethoxazole (Lot. 50204), Sulfathiazole (Lot. 50721), Sulfadiazine (Lot. 31010), Sulfadimethoxine (Lot. 40902), Sulfamethizol (Lot. 50225), Sulfamethoxypyridazine (Lot. 41001), Sulfapyridine (Lot. 101168). In order to eliminate the influence of the matrix, calibration through matrix blank sample was performed as well (EC 2017). Standard primary stock solution (1 mg/ml) was prepared in acetonitrile and stored at –20 °C. Standard mixtures in acetonitrile were also prepared from the primary stock solution for the calibration curves. All working solutions were prepared daily by serial dilution in 0.05 M anhydrous sodium acetate (pH 3.5). All the solution vials were wrapped in aluminum foil because of the sulphonamides drugs which are light-sensitive.

Sample Preparation

Meat samples were prepared according to the adapted QuEChERS method that was previously used (Novaković et al. 2017). The method involves extraction with acetonitrile in the presence of anhydrous magnesium sulphate and anhydrous sodium acetate. The sample (3 g) was measured and transferred into a centrifuge tube. After this, 3 ml of acetonitrile and 3 ml of water was added. After intensive stirring on a vortex, 3 g of anhydrous magnesium sulphate and 1 g of anhydrous sodium acetate was added. Exother-

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mic reaction occurred within 1 min after the intense stirring on vortex. The sample was then centrifuged at 1110 g for 5 min. 1 ml of upper acetonitrile extract was the transferred into a 5 ml tube, which contained 150 mg of anhydrous magnesium sulphate, 100 mg of primary and secondary amine and 50 mg of octadecyl (Anastassiades et al. 2003). The tube content was centrifuged for 5 min at 3000 rpm. After centrifuging, purified and clear extract was obtained. Then, 0.5 ml of the extract was evaporated under a nitrogen stream and reconstituted with 0.05 M anhydrous sodium acetate (pH 3.5). After that, derivatization step followed and 50 µL, 0.02 % fuorescamine in acetone was added. After 1 h of derivatization, the sample prepared in this way was ready to be analyzed for HPLC-FLD method (HPLC with fluorescence detector). Preparation of honey samples involves hydrolyzation with HCl solution (3 M, 800 µL) for 90 min and neutralization with citrate buffer (pH 3.5, 200 µL) and NaOH solution (10 M, 240 µL). The samples were derivatized with 0.2 % fluorescamine (200 µL) and the sample solution was passed through a 0.22 µm filter and injected to the on-line SPE-HPLC-FLD system (SPE – solid phase extraction).

Instrumentation and HPLC analysis

The analysis was performed on Thermo Scientific Ultramate 3000, (US) equipped with a binary pump and a fluorescence detector set at λex = 405 nm and λem = 495 nm. Separation of the compounds was achieved on Agilent ZORBAX Eclipse Plus C18 column (4.6 mm x 75 mm, 3.5 µm). The data obtained was processed using Chromeleon Software. Mobile phases were acetonitrile and deionized water with 2 % of acetic acid. The injection volume was 20 µL. Standards were prepared in blank matrix extracts, to counteract the matrix effect (SANCO 2013). Quantification was based on matrix calibration curves prepared from the standard solution of sulfonamide mix. The correlation coefficient (r^2) for all sulfonamides standard calibration plots were above 0.99. IBM SPSS Statistics 20 (IBM, Armonk, NY, USA) was used to determine the descriptive statistic parameters.

Method validation of sulfonamides

Sulfonamides determination method is an accredited method prepared in accordance with ISO 17025. Validation plan involved determination of precision, reproducibility, accuracy, linearity, LOQ, LOD and uncertainty (Tab. 1 and 2). The method precision was evaluated by repeatability using the honey and meat fortified with sulfonamide concentrations injected in triplicate (50.0 µg/kg, n = 20). The accuracy was calculated by recovery. Linearity was tested in a range from 50 to 1000 µg/kg, and was satisfactory in all ranges. Limit of detection (LOD – three standard deviations of the baseline noise) and limit of quantification (LOQ – then times the baseline noise standard deviation) were calculated using Excel. The LOD values ranged from 2 to 6 µg/kg and the LOQ varied from 6 to 21 µg/kg (Tab. 1 and 2). As chromatographic analyses alone without the use of spectrometric detection are not suitable as confirmatory methods in antimicrobial drug detection, HPLC method was used in this study only for screening purposes (EC 2002). The method used in this study was also used in PT (FAPAS 2018) where z score for sulfachlorpyridazine was – 1.2, and for sulfamerazine z was 0.8, indicating good results in sulfonamides measurement.

Results

The residues have been identified in six (3.3 %) honey samples (Tab. 3). The presence of sulfadiazine was established in four (2.2 %) samples, sulfamethizole in three (1.7 %) and sulfapyridine in two (1.1 %) samples. All honey samples originated from different producers. Positive finding prevalence according to honey type was as follows: flower 28.6 %, sunflower 10.0 %, meadow 3.8 % and acacia honey 1.7 %.

TABLE 1: *The average values of precision, reproducibility, accuracy, linearity, LOQ and LOD for sulfonamide residues in meat.*

Sulphonamide	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (r^2)	LOQ µg/kg	LOD µg/kg
Sulfadiazine	15.55	16.20	104.44	0.999	6	2
Sulfopyridine	7.25	12.62	98.95	0.996	6	2
Sulfathiazole	6.15	8.26	86.39	0.994	11	3
Sulfamerazine	15.11	14.56	95.16	0.999	7	2
Sulfamethazine	15.11	15.21	95.16	0.999	6	2
Sulfamethoxypyridazine	13.46	16.23	91.32	0.997	21	6
Sulfamethizole	9.87	10.21	91.17	0.999	11	3
Sulfachlorpyridazine	6.75	9.85	95.65	0.999	11	3
Sulfamethoxazole	11.89	11.62	93.87	0.999	18	6
Sulfadimethoxine	7.84	8.52	108.98	0.996	7	2

r^2: correlation coefficient

TABLE 2: *The average values of precision, reproducibility, accuracy, linearity, LOQ and LOD for sulfonamide residues in honey.*

Sulphonamide	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (r^2)	LOQ µg/kg	LOD µg/kg
Sulfadiazine	13.28	14.20	102.32	0.999	7	2
Sulfopyridine	8.13	10.46	98.95	0.996	7	2
Sulfathiazole	7.10	8.06	95.42	0.997	8	3
Sulfamerazine	10.07	12.21	90.26	0.999	7	2
Sulfamethazine	10.21	13.20	94.41	0.999	7	3
Sulfamethoxypyridazine	9.47	14.12	90.22	0.998	10	8
Sulfamethizole	7.80	11.95	97.10	0.999	11	3
Sulfachlorpyridazine	5.68	9.53	92.62	0.999	11	3
Sulfamethoxazole	9.87	10.45	90.71	0.999	9	3
Sulfadimethoxine	7.52	8.50	100.01	0.996	7	2

r^2: correlation coefficient

Sulfonamide residues were not detected in linden and forest honey. Compared to MRL values in the countries in which sulfonamides are allowed to be used in apiculture, four samples had higher values than MRL established for honey in Belgium (20 µg/kg), Switzerland and the UK (50 µg/kg). Three samples had extremely high total sulfonamide content, higher than MRL values set for honey in Brazil, exceeding even MRL values set for meat in EU (100 µg/kg).

For the purpose of evaluation of dietary exposure to sulfonamide residues through the intake of the contaminated honey found in this study, the daily intakes for consumers were estimated. The estimated daily intake – EDI (µg/kg bw per day) was calculated using the equation given by Kabrite et al. (2019):

$$EDI = \frac{\text{Residue concentration (µg/kg) x Daily intake of honey (50 g/person/day)}}{\text{Adult body weight (60 kg)}}$$

The EDIs were calculated using the dietary portion size of 50 g per person per day recommended by JECFA (Joint FAO/WHO Expert Committee on Food Additives) for honey (FAO/WHO 2009). EDIs for measured slufonamides ranged from 0.007 to 0.308 µg/kg bw per day, with an average of 0.072 µg/kg bw per day.

The residues were found in seven (6.1 %) meat samples (Tab. 4). Sulfapyridine was detected in six samples (5.2 %), sulfadiazine in two samples (1.7 %) and sulfamethizole values were not above LOQ. Residues were detected in 15.4 % poultry samples, 10.5 % beef and 3.6 % pork samples. All samples had residue values within MRL set for sulfonamides in meat. The samples were collected randomly, from a market which means that no presumptive positive samples were examined (samples from treated animals).

The total amount of examined samples included seven samples of flower honey, 13 poultry and 19 beef meat samples. A larger number of samples would have provided more realistic interpretation of these food types contamination.

TABLE 3: *Honey samples with quantified residue (µg/kg).*

sample	sulfadiazine	sulfamethizole	sulfopyridine	sum
sunflower	11	370	<LOD	381
meadow	<LOQ	59	<LOD	59
meadow	7.8	<LOD	<LOD	7.8
meadow	97	<LOD	9.1	106.1
flower	9.2	<LOD	8.2	17.4
acacia	<LOQ	208	<LOD	208
average	31.3	212.3	8.7	129.9
sd	43.9	155.5	0.6	143
min	7.8	59	8.2	7.8
max	97	370	9.1	381

sd: standard deviation

TABLE 4: *Meat samples with quantified residue (µg/kg).*

sample	sulfadiazine	sulfamethizole	sulfopyridine	sum
pork	6	<LOD	<LOQ	6
pork	<LOD	<LOQ	11.5	11.5
pork	<LOQ	<LOD	8.6	8.6
poultry	<LOQ	<LOD	7.4	7.4
poultry	<LOD	<LOD	9	9
beef	<LOD	<LOD	14.1	14.1
beef	11	<LOD	9.8	20.8
average	8.5	/	10.1	11.1
sd	3.5	/	2.4	5.1
min	6	/	7.4	6
max	11	/	14.1	20.8

sd: standard deviation

Discussion

Sulfonamides are the class of antimicrobial drugs most frequently detected in honey, together with tetracyclines (Reybroeck et al, 2010). Sulfonamides are used for the treatment of American and European Foulbrood, nosemosis and varoosis and are commonly administered by sugar syrup (Genersch et al., 2010; Dubreil-Chéneau et al., 2014).

Understanding the process of honey production is essential for interpretation of the occurrence of sulfonamide residues. Honey bees collect the nectar and excrete enzymes, which break down the nectar into simple sugars – glucose, fructose and sucrose (Solomon et al., 2006). When bees consume the nectar containing antimicrobial drugs, there is a lack of active drug metabolism as in other species such as mammals and birds. The drug is directly transferred to the nectar (Reybroeck et al., 2012). Bees deposit the nectar into the cells on the honeycomb. Under stable temperature conditions in the beehive (34 °C) the nectar evaporates into thick syrup (Solomon et al., 2006). This is aided by bees fanning it with their wings. Moisture evaporation increases the initial concentration of drugs in honey. Sulfonamides in honey create N-glycoside linkage with sugar molecules (Sajid et al, 2013). In the honey matrix, there is no time dependent depletion/elimination of residues as a result of pharmacokinetics, which is the case in mammal animals or poultry (Reybroeck et al., 2012). The content of residues in honey is predominantly affected by the properties of the residues. Contrary to tetracycline, tylosine and furazolidone which are unstable and undergo spontaneous chemical decay over time, sulfonamides are highly stable compounds that commonly remain intact. The concentration of residues in honey may depend on the honey yield (dilution effect), which depends on the production site (geographical area) and weather conditions at flowering time. Therefore, the specification of a withdrawal period, the interval between last treatment and harvest of honey, is extremely difficult (Reybroeck et al., 2012).

As the application of sulfonamides in apiculture is illegal and associated with random and uncontrolled dosage, the measured residue levels cannot clearly indicate the time of drug administration. It was established that concentrations of 180 000 µg/kg honey can be measured 2 weeks after the administration of sulfamethazine (Martinello et al., 2013). This is about 1000 times higher than maximum levels recorded in this research. The concentrations of about 1000 µg/kg can be found even a year after the drug application (Reybroeck et al., 2010). Sulfamethizole concentrations measured in this study suggested that the bees have been treated with sulfonamides, but contamination through wax is also likely. The migration of low levels of residues from the wax was confirmed in an *in vivo* research. The migration persists for at least three months (Martel et al., 2007; Reybroeck et al., 2010).

For the assessment of short-term dietary exposure, average EDI was compared to the acceptable daily intake (ADI). There is no ADI for measured sulfonamides so the results were compared with ADI values for sulfadiazine and sulfadimidine, set by JECFA (0–50 µg/kg bw per day) (FAO/WHO 2006). The average estimated daily intake (0.072 µg/kg bw per day) from this study showed that contribution of contaminated honey to dietary intake of sulfonamides was low. The short-term toxicological risk from contaminated honey is low, but there is a risk of allergy development in sensitive people. Sulfonamides are highly stable compounds that persist in the environment over long periods of time (Chen and Xie 2018). Thus, once contaminated honey remains unsafe for a long time. Sulfonamide residues cannot be removed from honey and long-term exposure to such residues is still unknown as a result of bioaccumulation. Since zero-tolerance level for sulfonamides in honey was set, all six samples with residue levels above LOQ are considered unsafe for human consumption (Tab. 3).

According to Bogdanov (2006), antibiotic residues are found more frequently in honey originating from third countries. Some 20–50 % of the honey imported in France, Belgium and Switzerland contained antibiotics, mostly streptomycin and sulfonamides, and tetracyclines and chloramphenicol, too. On the other hand, only 1 to 7 % of honey samples produced in Switzerland, Belgium and Germany had residues. Bogdanov (2006) reported that 2 to 7 % of Italian honey samples tested contained sulfonamides, tetracycline or tylosine. The results of this study revealed that honey contamination with residues in Serbia is in correlation with the average contamination frequency reported in Europe.

Unlike honeybees, the use of sulfonamides for cattle, pigs and poultry results in rapid drug distribution over the entire body. Sulfonamides are primarily metabolized in the liver, and in other tissues as well (Patel and Welling 1980). Parent drug and metabolite excretion occurs through the kidneys. Withdrawal period depends on sulfonamide type, dosage and animal species ranging from 4 to 7 days (Hassan et al., 2014; Khatun et al., 2018). Although the presence of sulfonamides was confirmed in 6.1 % of meat samples, the levels which threaten human health (i.e. above 100 µg/kg) were not detected in any of the examined samples from Serbian market (Tab. 4).

The comparison of residue levels in honey and meat reveals some major differences. They are the following: the average sulfonamide concentration in honey was 10 times higher than that in the meat, 50 % of honey samples containing residues had sulfonamide levels above 100 µg/kg, whereas residue levels determined in all meat samples did not exceed 21 µg/kg. However, statistical data analysis (t-test) did not reveal statistically significant difference between residue levels in meat and honey ($p = 0.11$), which is due to highly variable sulfonamide contents in honey (ranging from 7.8 to 381 µg/kg). Statistically significant difference would probably be determined if a larger number of positive samples was used.

Conclusions

The results of this research revealed that officially approved use of sulfonamides enables appropriate control of residue levels in foodstuffs by applying prescribed dosage over prescribed period of time. Consequently, compliance with the recommended withdrawal period prevents unsafe food coming to the market. Unlike in pig, cattle and poultry farming, sulfonamides are prohibited in apiculture. Thus, beekeepers do not have possibility to use the authorized drug at prescribed dosage and they administer the therapy randomly, probably using high doses over a prolonged period. Honeybees are highly specific food producing animals, characterized by a lack of active metabolism of antimicrobial drugs, which causes consequent deposition of drug residues in the honey. Antimicrobial elimination occurs only by spontaneous biodegradation during storage. Sulfonamides are poorly biodegradable and highly stable in honey. Thus, once contaminated, honey remains unsafe for consumption during several months. Extreme values of sulfonamides measured in honey samples and low average EDI indicate that toxicological risk from short-term exposure is low. However, there is a significant risk from allergic reactions in sensitive people. There is also a risk from long-term exposure and bioaccumulation with possible influence on human microflora and transfer of resistance genes.

The results of this study clearly indicate that compliance with the good farming practices and appropriate use of veterinary drugs in primary production can ensure safe foodstuff products, while illegal practices severely affect the safety of honey as a highly valuable and nutrient and rich foodstuff.

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Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials discussed in this manuscript.

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