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Distribution of Sulfathiazole in Honey, Beeswax, and Honeybees and the Persistence of Residues in Treated Hives

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ABSTRACT: This study was performed to evaluate the distribution and depletion of sulfathiazole in different beehive matrices: honey, honeybees, "pre-existing" honeycomb, "new" honeycomb, and capping wax. Sulfathiazole was dissolved in sugar syrup or directly powdered on the combs, the matrices were sampled at different time points, and sulfathiazole residues were quantified by high-performance liquid chromatography with fluorescence detection. In honey, the higher concentration of sulfathiazole (180 mg kg^{-1}) occurred 2 weeks after the last treatment in syrup. In beeswax, drug concentration was higher than in honey, particularly with powder administration, with a maximum level (340 mg kg^{-1}) 3 days following the last treatment. The strongest contamination in honeybees (28 mg kg^{-1}) was achieved with sulfathiazole administered in powder 3 days after the second treatment. The high persistence of sulfathiazole in the different beehive matrices suggests that it could be a reliable marker of previous treatments performed by beekeepers.

KEYWORDS: *honey, honeybees, beeswax, residues, sulfathiazole*

INTRODUCTION

Sulfonamides play an important role as effective chemotherapeutics of bacterial and protozoal diseases in veterinary medicine. In food-producing animals, residues are depleted with high variable velocity depending on many factors such as the nature of the compound, its formulation and route of administration, the treated animal species, and genotypes.[1]

Sulfonamides are synthetic antimicrobial agents that are sometimes used in apicultural practice for the treatment and control of American foulbrood (AFB), one of the most widespread and devastating diseases of honeybee broods.[2,3] Sulfonamides, as other antimicrobials, are effective against the vegetative form of the spore-forming causative agent, *Paenibacillus* larvae, but ineffective against its spores. Some publications quote that sulfa drugs could be effective against other bee pathogens such as *Nosema* spp.[4] Generally, the therapeutic use of sulfonamides in veterinary medicine is allowed in livestock production in the European Union (EU), but there are maximum residue limits (MRLs) of 100 $\mu g\ kg^{-1}$ in muscle, fat, liver, and kidney of all animal species and 100 $\mu g\ kg^{-1}$ in milk from cattle, sheep, and goats.[5] No authorized antimicrobial veterinary medicines intended for apiculture are available within the EU. Furthermore, sulfonamides can be used in apiculture based on the cascade system, as described in Directive 2004/28/EC[6] and Regulation (EU) n. 37/2010,[5] upon prescription of a veterinarian but with zero tolerance for residues. Nevertheless, their residues are often found in commercial honeys and in other beehive products.[7]

In the triennium 2008−2010 Rapid Alert System for Food and Feed (RASFF) reports, 12 of 53 communications (23%) about drug residues in beehive products were attributable to sulfonamides.[8]

In Italy, the National Plan for the control of veterinary drug Residues (PNR) established the assessment of the presence of sulfonamides in honey, and it admits an action limit of 5 $\mu g\ kg^{-1}$. The Italian Ministry of Health considers concentrations higher than this limit as results of illegal treatments.[9]

Besides honey, beeswax is also an important bee product, considering that contaminated beeswax could act as a vector of honey contamination with antimicrobial residues.[10] There are few studies describing the residual distribution of antimicrobials in beehive matrices (not only honey), for example, tetracyclines,[11,12] lincomycin,[13] and mirosamicin,[14] but sulfonamides have never been studied.

This study evaluated the distribution and depletion of one of the most commonly used antimicrobials, sulfathiazole, as evidenced in RASFF reports.[8] Sampling was performed at different time points and in different beehive matrices: honey, honeybees, "pre-existing" honeycomb, "new" honeycomb, and capping wax. Consolidated beekeeping practices were taken into considertaion, so the beehives were treated in two ways: once a week for 3 weeks sulfathiazole was either dissolved in sugar syrup to feed honeybees or powdered directly on the combs. The different matrices were sampled, at different time points, for 12 months following the last treatment.

The analytical method used was based on those of Schwaiger et al.[15] and Thompson et al.[16] It consisted of a liquid−liquid extraction and an HPLC−fluorometric determination after precolumn derivatization with fluorescamine.

MATERIALS AND METHODS

Bee Colonies and Treatments. Four *Apis mellifera ligustica* hybrid (*ligustica* × *carnica*) colonies were housed in Dadant−Blatt hives composed of eight combs and one wax foundation ("new" honeycomb), with an inner syrup feeder, located near Bassano del Grappa (Vicenza, Italy) (45° 46′ 52.00″ N, 11° 40′ 34.42″ E, 329 m

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asl). Two control colonies (blank) were located 5 km from the hives under investigation to reduce the risk of cross-contamination due to drifting or robbing, but the landscape of the two areas was homogeneous. The colonies used in this study were homogeneous in terms of strength and showed no clinical signs of European or American foulbrood.

Two colonies were treated with 1 g of sulfathiazole dissolved in 500 mL of aqueous sucrose solution (50%, w/v) and poured into the feed compartment once a week for 3 weeks. Two colonies were treated with a powder mixture composed of 1 g of sulfathiazole and 10 g of sucrose, which was sprinkled on the top bar of the honeycombs, also once a week for 3 weeks. The two control colonies were fed untreated sucrose solution at the same time points.

The trial started in June 2010, when the nectar flow was sufficient to build up the new combs, and ended in June 2011.

Sampling. The samples collected from each hive were uncapped honey, taken from three different points in the hive (about 10 g); adult honeybees (about 10 g); "pre-existing" honeycomb, which was already present in the hive (approximately 5 × 5 cm); "new" honeycomb, generated from a new foundation inserted in the hive at the beginning of the experiment (approximately 5 × 5 cm); and capping wax (5−6 g). Honey was separated mechanically from beeswax by manual pressure.

Samples were collected before the trial, on the day of the second and third treatments (just before drug administration), 3 and 7 days after each treatment, at 2 and 3 weeks after the last treatment, and at 1, 2, 3, and 12 months after the last treatment. The sampling time points are summarized in Table 1.

Table 1. Experimental Design for Beehive Treatments and Sample Collection

timeline	treatment	sampling
0		1
7th day	1st	
10th day		2
14th day	2nd	3
17th day		4
21st day	3rd	5
24th day		6
28th day		7
35th day		8
42nd day		9
72nd day (1 month after last treatment)		10
102nd day (2 months after last treatment)		11
132nd day (3 months after last treatment)		12
402nd day (12 months after last treatment)		13

All samples were stored at −20 °C until analysis.

Materials and Reagents. Apart from honey, samples were homogenized with a grinder, cooled with liquid nitrogen (A11 basic IKA-Werke GmbH & Co. KG, Staufen, Germany). Simultaneous shaking of the test tubes was achieved using a table shaker Multi Reax (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). A Universal 32R centrifuge (Hettich GmbH & Co. KG, Tuttlingen, Germany) and a microprocessor pH meter (Hanna Instruments, purchased by Vetrotecnica, Padova, Italy) were used for sample preparation. Cleanup was performed with Supelco DSC-SCX solid phase extraction (SPE) cartridges (500 mg/3 mL) purchased from Sigma-Aldrich (Milan, Italy).

All solvents used for sample preparation (chloridric acid, sodium hydroxide, aceton, dichloromethane, acetic acid, methanol, ammonium hydroxide) were of HPLC grade, purchased from either Sigma-Aldrich or Prolabo (VWR International, Milan, Italy). Fluorescamine, citric acid, sodium acetate, and sodium chloride were purchased from Sigma-Aldrich. Water class 3 for laboratory use[17] produced by a Milli-Q water purification system was used.

Sulfathiazole (Fluka, 98% purity) was used for hive treatments, high-purity sulfathiazole (Vetranal, 99.9% purity) was used as the analytical standard, and sulfamonomethoxine (Sigma, 98.5% purity) was used as an internal standard. These were purchased from Sigma-Aldrich.

Sulfathiazole analysis was performed by HPLC coupled with a fluorescence detector (Shimadzu, Tokyo, Japan). Separation was obtained using a Supelco Ascentis Express C8 column (150 mm × 3 mm, particle size = 2.7 μm) with a C8 guard column installed (Sigma-Aldrich).

Analytic Procedure: Honey Samples. Five grams of each honey sample was weighed in a polypropylene tube and chloridric acid (5 mL, 2 mol L^{-1}) added. The honey was dissolved completely by manual shaking and an automatic stirrer (10 min). Sodium hydroxide (2 mL, 5 mol L^{-1}) was added, and the pH was adjusted to between 5 and 7 with sodium hydroxide (0.5 mol L^{-1}) or chloridric acid (0.1 mol L^{-1}). A solution of acetone/dichloromethane (80:20 v/v, 10 mL) and 2 g of sodium chloride were added, and the mixture was shaken for 10 min. The liquid phases were separated by centrifugation (4500*g*, 10 min). The organic phase (5 mL) was transferred to a graduated glass cylinder and evaporated to 0.5 mL under a stream of nitrogen. The volume was adjusted to 1 mL with water, filtered through a 0.45 μm cellulose syringe filter, and injected for HPLC analysis.

All samples collected from treated hives were diluted 1:4000 before analysis. If the sulfathiazole concentration detected was lower than the concentration range of the method (Table 2), the samples were processed again and not diluted.

Analytic Procedure: Beeswax and Honeybee Samples. Two grams of each beeswax or honeybee sample, previously homogenized with liquid nitrogen cooling, was weighed in a polypropylene tube, and a solution of acetone/dichloromethane (50:50 v/v, 20 mL) was added. The beeswax was completely dissolved by heating tube in a water bath (60 °C, 5 min) and stirred (5 min), and then the tubes were cooled in a freezer (−20 °C, 10 min).

Honeybee samples did not require any heating and were directly shaken for 5 min after addition of the extraction solution. The samples were then centrifuged (5000*g*, 10 min), and the liquid phase was filtered through filter paper into a new tube. Proper dilutions were performed only in samples from treated hives, not in control ones: wax samples were diluted 1:7000 and honeybee samples 1:600 with extraction solution. If the sulfathiazole concentration detected was lower than the concentration range of the method (Table 2), the samples were processed again and not diluted.

Five milliliters of this sample solution was added to glacial acetic acid (250 μL per tube) and loaded into SCX cartridges previously conditioned with a solution of acetone/dichloromethane/acetic acid (47.5:47.5:5 v/v, 10 mL) and then washed with water (3 mL) and methanol (5 mL). Sulfathiazole was eluted with ammonia solution (2.5%) in methanol (6 mL), and the eluate was evaporated to dryness under a stream of nitrogen at 50 °C. The sample was then reconstituted with 1 mL of reconstitution phase (70% acetic buffer,

Table 2. Method Performance Parameters for Sulfathiazole Detection during This Study

matrix	concn range (μg kg^{-1})	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	repeatability (%)	recovery (%)	selectivity (%)
honey	2−50	1	2	19	70	<1
beeswax	2−50	1	2	25	86	<1
honeybees	2−50	1	2	13	70	<1

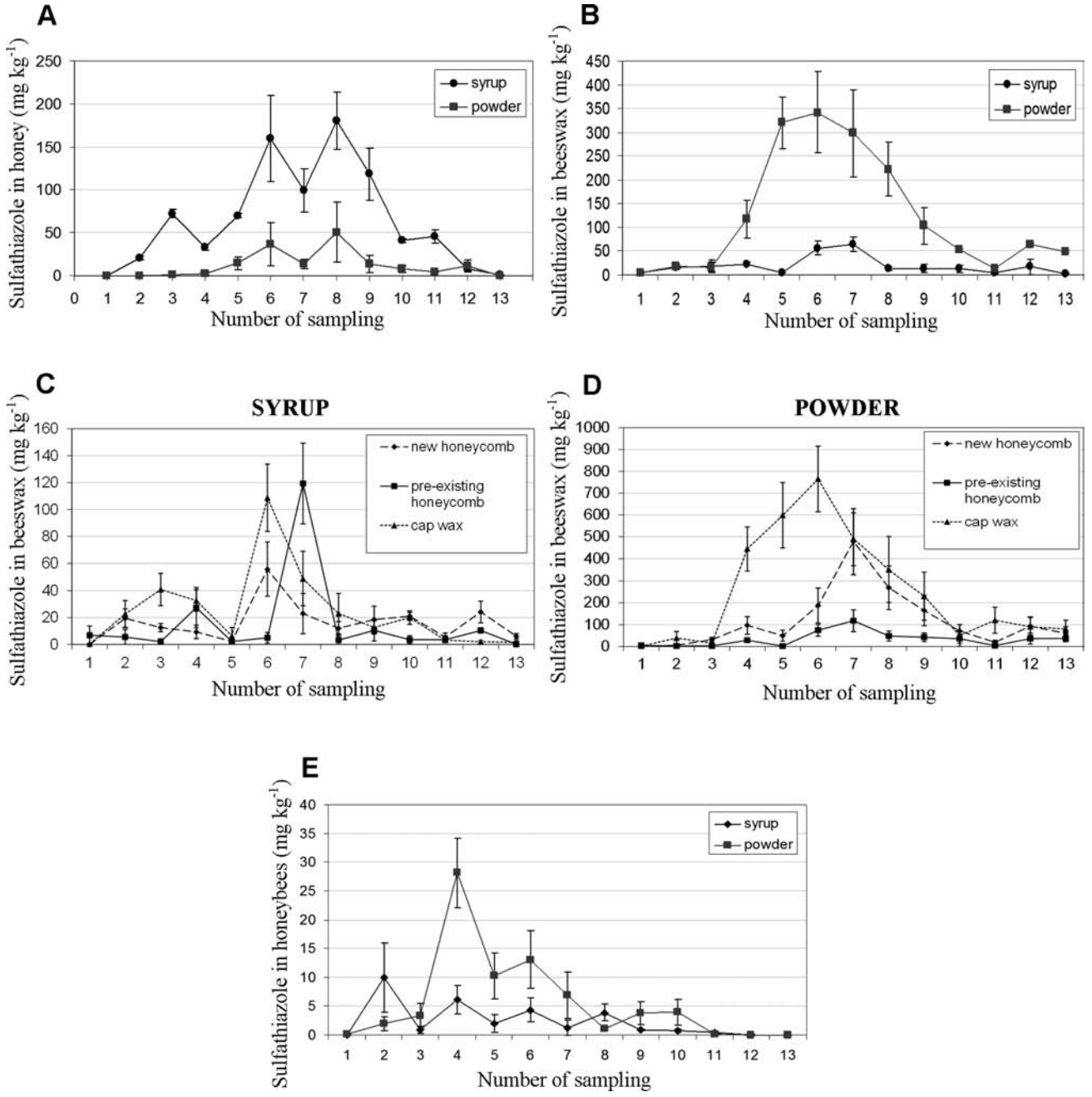

Figure 1. Mean concentrations of sulfathiazole in honey after syrup treatment and powder treatment, $n = 6$ (A); mean concentrations of sulfathiazole in beeswax after syrup treatment and powder treatment (error bars quantified by weighted standard deviation calculation), $n = 18$ (B); distribution of sulfathiazole after syrup treatment in beeswax matrices: new honeycomb, pre-existing honeycomb, and capping wax, $n = 6$ (C); distribution of sulfathiazole after powder treatment in beeswax matrices: new honeycomb, pre-existing honeycomb, and capping wax, $n = 6$ (D); mean concentrations of sulfathiazole in honeybees after syrup treatment and powder treatment, $n = 6$ (E).

27% acetonitrile, and 3% methanol), filtered through 0.45 μm cellulose syringe filter, and injected for HPLC analysis.

Preparation of Standard Solutions. Standard solutions of sulfathiazole (1 mg L^{-1}) and sulfamonomethoxine (1 mg L^{-1}) were prepared in methanol and stored at −20 °C for 1 month in dark glass bottles.

Recovery Studies. Spiked samples were used to evaluate method recovery. Sulfathiazole (5 μg kg^{-1} for honey, 50 μg kg^{-1} for beeswax and honeybees) was added to blank matrices prior to extraction. The recovery of sulfathiazole in several assays, calculated with an internal standard method, and other performance parameters of the method are shown in Table 2. The number of replicate measurements varied between 10 and 15.

Quantification. The internal standard method was used to obtain more reproducible results, and sulfamonomethoxine was added during reconstitution at a final concentration of 25 μg kg^{-1}. Residue concentrations were calculated by using solvent-based calibration curves, and the results were recovery corrected (recoveries experimentally determined at each work session).

Sample Analysis. The samples were subjected to precolumn automatic derivatization: 100 μL of sample was mixed with 300 μL of derivatization reagent (100 μL of fluorescamine 0.1% w/v in acetone and 200 μL pf citrate buffer 1 mol L^{-1}, pH 3) and were incubated for 20 min in the autosampler, prior to injection. The injection volume was 50 μL, with a flow rate of 0.6 mL min^{-1}, and the column temperature was set at 34 °C. The mobile phase employed was a

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Table 3. Comparison of Maximum Contamination Detected and Persistence of Sulfathiazole 12 Months after the Last Treatment (Concentrations Expressed as Mean Values, $n = 6$)

		honey	new honeycomb	pre-existing honeycomb	capping wax	honeybees
syrup	max contamination (mg kg^{-1})	180 ± 33	55 ± 20	119 ± 30	109 ± 27	10 ± 6
	persistence after 12 months (mg kg^{-1})	0.9 ± 0.06	6 ± 2	1 ± 0.5	2 ± 0.9	0.02 ± 0.01
powder	max contamination (mg kg^{-1})	51 ± 35	479 ± 151	118 ± 48	765 ± 147	28 ± 6
	persistence after 12 months (mg kg^{-1})	0.05 ± 0.01	60 ± 29	37 ± 14	80 ± 38	0.02 ± 0.01

mixture of acetate buffer (0.125 mol L^{-1}), methanol, and acetonitrile used in a ramp mode starting with 70% acetate buffer, 27% acetonitrile, and 3% methanol and arriving at 13.5 min to 64% acetate buffer, 33% acetonitrile, and 3% methanol. The mobile phases returned to the starting condition in 30 s and were maintained for 3.5 min at the end of acquisition. Wavelengths selected were 400 nm for excitation and 490 nm for acquisition.

RESULTS AND DISCUSSION

No residues of sulfathiazole were detected in any samples (honey, beeswax, and honeybees) collected before, during, and after treatments in control hives, confirming that no cross-contamination between control and treated hives occurred. Furthermore, these data evidenced that not even contamination of environmental origin was present during the study.

In honey samples from the treated hives, the highest concentration of sulfathiazole (180 mg kg^{-1}) was achieved with the syrup, 2 weeks after the last treatment (sampling 8). Persistence of the residue was observed 12 months after the last treatment with sulfathiazole (0.9 mg kg^{-1}). The same concentration trend was shown in powder-treated hives, but the highest concentration peak was 50 mg kg^{-1}, 3 times less than concentrations following treatment with sulfathiazole in syrup (Figure 1A). The last sampling, 12 months after the final treatment, revealed minimal sulfathiazole residue in powder-treated hives (0.05 mg kg^{-1}). The higher contamination of honey sampled from hives treated with sulfathiazole in syrup rather than powder suggested that honeybees ingest most of the drug just after the treatment and rapidly eliminate it. Previous studies demonstrated that drug elimination could occur through metabolism, advection, and deposition.[12,18] This could be also confirmed by the rapid decrease of sulfathiazole concentration in honeybee samples after the last treatment. The rapid incorporation of sulfathiazole in honey is also due to the fast removal and storage of syrup by honeybees in comb cells.

Beeswax sample concentrations are shown in Figure 1B calculated as mean values obtained from the different wax matrices analyzed (capping wax, pre-existing honeycomb, and new honeycomb). The mean drug concentration was higher than in honey samples, particularly following powder administration, with a maximum level (340 mg kg^{-1}) 3 days after the last treatment (sampling 6). A high persistence of contamination was observed, 12 months after the last treatment, and drug concentration was 48 mg kg^{-1}. The difference between sulfathiazole mean concentration in powder- and syrup-treated hives was higher than in honey samples: the maximum amount of sulfathiazole achieved in syrup-treated hives (64 mg kg^{-1}) occurred 1 month after the last treatment (sampling 7) and was 5 times lower than following powder treatment.

Figure 1, panels C and D, shows the distribution of sulfathiazole in the different types of wax collected (new honeycomb, pre-existing honeycomb, and capping wax) related to the different types of drug administration. Larger amounts of sulfathiazole were detected in beeswax samples in hives treated with powder drug, which was sprinkled on the top bar of the honeycombs, because of the best spreading of the drug compared with syrup that was confined in the feeder compartment and diffused only by honeybee activities within the hive.

By comparison of the different wax matrices it was observed that capping wax was the most contaminated one. Capping wax is the most exterior part of the comb as well as the one that is constructed during the treatment period, so it is the most exposed during powder administration. In fact, we suppose that part of the sulfathiazole could be embedded within the wax. Between pre-existing and new honeycombs the highest concentration of sulfathiazole was detected in new honeycombs that were built in the beehive during the experimental period and were therefore more subjected to drug contamination.

Honeybees were the matrix with lowest contamination. The strongest contamination (28 mg kg^{-1}) occurred following powder administration of sulfathiazole, 3 days after the second treatment (sampling 4), and a rapid decrease in residue concentration occurred after the last treatment (Figure 1E).

The values of maximum contamination calculated during the study and the residual amount 12 months from the last treatment in the different matrices analyzed are summarized in Table 3.

Sulfathiazole depletion profile could depend on the sampling that was performed at different points within the hive or on the different availability of food for the honeybees in their natural surroundings beyond the syrup administered for the study. However, the data obtained from honey and honeybees in syrup-treated hives showed a parallel discontinuous trend of drug concentrations, and we observed an increase of sulfathiazole concentration after each treatment in honeybees (Figure 1E), but after a week in honey (Figure 1A). In samples collected from powder-treated hives, sulfathiazole depletions in honey and honeybees are different. Indeed, we observed a drug accumulation in honey only following the last treatment (Figure 1A) and in honeybees after each treatment (Figure 1E), so we can hypothesize an indirect contamination of this last matrix.

In conclusion, the treatment with sulfathiazole in syrup determined a higher honey contamination, whereas the powder administration caused a stronger contamination of beeswax samples. The wax matrix most contaminated in the experiment was capping wax, and by comparison of pre-existing and new honeycombs, the highest concentration of sulfathiazole was detected in new honeycombs.

The high persistence of sulfathiazole in the different matrices suggests that it could be a valuable marker for past treatments.

According to our study, beeswax was the most contaminated matrix after powder treatment and, together with the high persistence, it could be a potential risk for consumers' health because of the use of beeswax in the food (as a component in

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chewing gum, candy, bakery, etc.), pharmaceutical, and cosmetic industries.[18] In addition, the practice of reuse of beeswax by beekeepers could cause the contamination of "new" produced honey during the next honey season, due to the migration of the sulfonamide from contaminated beeswax to honey. According to beekeeping practice, beeswax undergoes different kinds of processes to make it reusable, particularly treatments with high temperature in water, high-pressure steam, and straining, that could cause a deterioration of sulfathiazole or, in general, of contaminants. A future perspective could be the evaluation of the consequences of these rendering processes on the amount of drugs left in contaminated beeswax.

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Notes

The authors declare no competing financial interest.

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