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Research Article

Sulfonamide Residues: Honey Quality in the Czech Market

Sandra Dluhošová, Ivana Borkovcová, Lenka Kaniová, and Lenka Vorlová

Department of Milk Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Palackého Tř. 1946/1, 612 42 Brno, Czech Republic

Correspondence should be addressed to Sandra Dluhošová; sandradluhosova@seznam.cz

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In this study, we determined four sulfonamide compounds (sulfadiazine, sulfadoxine, sulfamethoxazole, and sulfathiazole) in honey marketed in the Czech Republic (Brno). The content of these compounds was monitored in 50 honeys with different botanical and geographical origin. Preanalytical treatment included acidic hydrolysis with 10% trichloroacetic acid (TCA) and double extraction with acetonitrile and dichloromethane. Chromatographic analysis was performed by ultraperformance liquid chromatography with tandem mass spectrometry (UPLC-MS/MS) using a triple quadrupole (QqQ) in positive ionization mode (ESI^+). For separation, the mobile phase was a 0.05% aqueous solution of formic acid (A) and 0.05% formic acid in methanol (B) with a gradient elution of 0–3 minutes: 10% B; 3–3.6 min: 90% B; and 3.6–5 min: 10% B, flow rate 0.30 ml/min. Three positive samples (6%) were found containing sulfadiazine (232.88 and 618.87 μg/kg) and sulfamethoxazole (62.64 μg/kg). According to the country of origin, two of these honeys were from European Union (EU) countries, and one was labeled as a blend of honey from EU and non-EU countries.

1. Introduction

Consumers regard honey as a wholesome natural product, and as such, there is an expectation of quality and the resultant health benefits. The high price is mainly due to seasonality and the limited availability of honey in the market as a result of decline in hive strength from disease, environmental pollution, or sudden changes in weather in any given year. Honey is therefore a very frequent target for food adulteration. The same is true in the case of noncompliance with legislation related to antibiotic use on bees.

Sulfonamides are broad-spectrum synthetic bacteriostatic agents used against Gram-positive and Gram-negative bacteria as well as protozoans in human and veterinary therapy. These drugs are very good therapeutic agents and are easily accessible, and that led to its overuse in livestock. The result has been sulfonamide residues in foods of animal origin (milk, meat, eggs, fish, or honey). This is a potential hazard for consumers in terms of the development of resistant microorganisms, allergic reactions, or toxic effects [1, 2]. One specific application of sulfonamides is their use on bees. In 1944, Haseman and Childers attempted to use sulfathiazole in the prevention of American foulbrood (*Paenibacillus larvae*). Over the years, the use of sulfonamides has also been tried against European foulbrood (*Melissococcus plutonius*) and recently in the fight against nosemosis (*Nosema apis*, *N. ceranae*) following the scarcity of fumagillin in the EU. Sulfonamides are also often used in combination with diaminopyrimidines (trimethoprim). A preparation with sulfathiazole sodium as the active ingredient was registered for the control of American foulbrood, and despite the apparent efficacy of these drugs, the alarming presence of antibiotic residues in honey led to the withdrawal of this registration in the 1970s [2–8]. The use and the presence of pesticide residues in the environment could have been because of colony collapse disorder (CCD). This also has a knock-on effect in the yields of agricultural crops, where the role of bees as pollinators is irreplaceable [9].

There are currently no maximum residue limits (MRLs) in the EU for sulfonamides (and other veterinary medicinal products) in honey, and treatment of hives with these

antimicrobials is prohibited [10, 11]. But European legislation cannot govern the behavior of producers that trade in honey, and so its quality often does not meet the prescribed requirements, specifically with regard to the illegal use of antibiotics and other chemotherapeutics in the EU. The issue also concerns countries outside the EU, where the treatment conditions for bee colonies are quite different. Honey from these countries then enters the EU market. In Switzerland, MRLs for honey are set at 50 μg/kg for total sulfonamides, whereas in China, the same value is set for each sulfonamide separately [5]. Each EU state and National Reference Laboratory facilitates the trade in honey by monitoring these substances as part of a monitoring plan and supervising compliance. Despite this, cases with residues are still reported. The Rapid Alert System for Food and Feed (RASFF) ensures the prompt reporting of food residues and withdrawal of any unidentified or dangerous product from the market in all EU countries [12, 13].

The preanalytical protocol for food sample preparation and a sufficiently sensitive analytical method must be chosen depending on the requirements arising from the legislation. Most studies use acidic hydrolysis (with hydrochloric acid, trichloroacetic acid, or citric acid) as an essential step in the determination of sulfonamides in honey. This converts the analytes to the free form and is used in combination with sonication, tempering, and extraction. In multiresidue analysis, consideration is given to the antibiotic family group and their physicochemical properties, and McIlvaine buffer is applied along with a chelating agent (disodium salt of ethylenediaminetetraacetic acid, EDTA). SPE cartridges with a wide pH range or ion-exchange columns (Oasis HLB, Sep-Pak C18, SCX, Bond Elut) are often used for solid-phase extraction (SPE) in varied conditions (online/offline). Liquid-liquid extraction (LLE) often makes use of acetonitrile in combination with dichloromethane. The predominant separation method for determination of sulfonamides in samples is liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS). Fluorescence detection following pre- or postcolumn derivatization with fluorescamine is also used [1, 8, 14–17].

The aim of this study was to evaluate the current status of sulfonamide residues in honey available in the first quarter of 2018 in the market in the Czech Republic, an EU member state, and to assess compliance with European legislation.

2. Materials and Methods

2.1. Standards and Chemicals. All standards and chemicals used in this study were analytical grade. Sulfonamide (SAs) standards: sulfadiazine (SDZ), sulfadoxine (SDX), sulfamethoxazole (SMZ), and sulfathiazole (STZ) were all 99.9% (Sigma-Aldrich, Germany); acetonitrile and methanol for LC-MS and dichloromethane for HPLC (Merck Germany); 98–100% formic acid (Sigma-Aldrich, Germany); trichloroacetic acid, p.a., and sodium hydroxide (Penta, Czech Republic); argon (Linde Gas, Germany). Water for LC analysis was distilled and deionized using Aqua Osmotic 03 (Czech Republic).

2.2. Instrumentation. The following equipment was used in our study. Analytical balances from Kern Analytical Laboratory (Balingen, Germany); ultrasonic bath (Kreintek, Slovakia); water bath GFL 1002 (GFLmbH, Germany); orbital shaker GFL 3005 (Merci, Czech Republic); pH Meter 211 Microprocessor (Hanna Instruments, Romania); Hermle centrifuge, type Z 326 K (Labortechnik GmbH, Germany); vacuum pump ME 2NT (Vacuumbrand, Wertheim, Germany); vacuum rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland); Acquity H-Class UPLC separation module with triple quadrupole mass detector (QqQ) (Waters, USA); Acquity UPLC BEH C18 chromatographic column 2.1 × 100 mm, particle size 1.7 μm (Waters, Ireland); nitrogen generator (Parker, UK); and vacuum pump Sogevac SV 40 BI (Leybold GmbH, Germany). The results were processed using MassLynx 4.1 software (Waters, USA).

2.3. Honey Samples. In this monitoring study, 50 honeys of different botanical and geographical origin were purchased from Czech retailers. The samples were classified by region of origin into three groups: honey from EU countries (*Group 1*, $n = 25$), blends of honey from EU and non-EU countries (*Group 2*, $n = 20$), and honey from outside the EU (*Group 3*, $n = 5$).

2.4. Standard Solutions. Standard stock solutions of SAs were prepared by dissolving in methanol; the concentration of the stock solutions was 0.4 g/l, and they were stored at 4 ± 2°C for at most one month.

Working solutions of SAs for the model study were prepared in three concentrations: 0.5, 1.0, and 5 μg/l. Each concentration of working solutions was prepared in 12 replicates and processed according to Section 2.5.

2.5. Sample Preparation. Samples were processed using a modified version of the protocol published by Verzegnassi et al. [18]. 2 g of honey was weighed and dissolved in 10 ml of 10% TCA. To release the sulfonamide bonds from the honey matrix and for more efficient extraction, the solutions were sonicated for 15 minutes and tempered in a water bath at 64°C (1 hour). After cooling, the pH of the sample was adjusted to 6.5 using 1M and 0.1M NaOH. For dual liquid-liquid extraction (LLE), acetonitrile (8 ml), and dichloromethane (2 ml) were added. The mixture was shaken thoroughly on a shaker for 5 minutes and centrifuged at 5°C at 5000 rpm for 10 minutes. The organic layer was transferred to a ground flask. The solvent was evaporated on a vacuum rotary evaporator at 55°C to dryness. The sample was reconstituted in 1 ml of mobile phase A (0.05% formic acid), and filtered into a vial and analyzed.

Matrix calibration solutions processed using the same procedure as samples were diluted from stock solutions at a concentration range of 0.1–100 μg/l (0.1; 0.5; 1; 5; 10; 20; 50; 100 μg/l).

2.6. Chromatographic Conditions. Sulfonamides were analyzed on an Acquity H-Class UPLC. For separation, an Acquity UPLC BEH C18 column 2.1 × 100 mm with 1.7 μm particle size was used. MassLynx 4.1 software was used for data processing, quantitative evaluation, and confirmation. The mobile phase was a mixture of water (A) and methanol (B), each containing 0.05% formic acid. The gradient was established as follows: 0–3 min, 10% B; 3–3.6 min, 90% B; and 3.6–5 min, 10% B. The flow rate was 0.3 ml/min, column temperature was 35°C, injection volume 5 μl, and the run time was 5 minutes. The measured parameters for individual analytes are given in Table 1.

2.7. Mass Spectrometry Detection Parameters. Direct infusion of analytes was used to optimize the mass detector, and the concentration of each analyte was 2 mg/l. The precursor ions for SDZ, SDX, SMZ, and STZ (311; 251; 254; and 256 m/z respectively) were monitored in MS full scan. The subsequent MS/MS daughter scan revealed product fragments at 156, 108, and 92 m/z that correspond, respectively, to $[M + H\text{-}RNH_2]^+$, $[M + H\text{-}RNH_2\text{-}SO]^+$, and $[M + H\text{-}SO_2]^+$. All sulfonamides had the same ion fragments (156, 108, and 92 m/z) arising from the same structural skeleton.

Mass detection was performed on a tandem quadrupole (QqQ) in positive electrospray ionization mode (ESI^+); the mobile phase was acidified with formic acid to generate protonated molecules. The other parameters were extractor voltage 3 V, RF voltage 0.1 V, source temperature 120°C, desolvation gas temperature 350°C, desolvation gas flow rate 650 l/h, nebuliser gas flow rate 50 l/h, collision gas flow (argon) 0.2 ml/min, and collision pressure 4.10^{-3} mbar.

Multiple reaction monitoring (MRM) was applied, and two product ions (for quantification and confirmation transition) were selected for each sulfonamide. The mass detector settings and gradient values for individual analytes are shown in Table 2.

3. Results and Discussion

The issue of pharmaceutical residues in honey has attracted attention over the past few years and is being closely followed by both the professional and lay public. Monitoring is ensured by the regulatory authorities of the states concerned. Through the RASFF portal, it is possible to trace possible misgiving from suppliers and producers of honey. Dmitrienko et al. [19] reported a range of antibiotic residues in foods: 20% sulfonamides, 19% fluoroquinolones, 15% aminoglycosides, 15% amphenicols, 15% beta-lactams, 8% oxazolidinones, and 8% tetracyclines. In 2009–2013, the incidence of antimicrobial residues was 71%, of which up to 35% was for sulfonamides followed by tetracyclines (15%), nitrofurans (13%), lincosamides (13%), aminoglycosides (10%), nitroimidazoles (8%), macrolides (5%), and quinolones (3%) [16]. It is clear that sulfonamides are still one of the first choices in antibiotic treatment. In the decade from 2008 to 2018, 78 reports of residues of veterinary pharmaceuticals in honey and royal jelly were submitted to the RASFF system, of which 23 cases (29%) were sulfonamides. Concentrations in the positive samples ranged from 3 to 865 μg/kg. The sulfonamides found were sulfathiazole, sulfamethoxazole, sulfamethazine, sulfadimidine, sulfadimethoxine, and sulfadiazine [13]. Three cases of sulfonamide (sulfathiazole and sulfadimidine) residues in honey from Ukraine and Moldavia have been reported in the Czech Republic. Apart from the Czech Republic, other European countries have recorded sulfonamide residues in honey from Turkey, China, Poland, Germany, Mexico, Belgium, Portugal, Lithuania, Bulgaria, Hungary, Egypt, and Serbia [13]. The Czech Agriculture and Food Inspection Authority (CAFIA) has also recorded cases where honey contained residues of the aminoglycoside antibiotic streptomycin and chloramphenicol in addition to sulfadimidine and sulfathiazole [20–22]. Among the sulfonamides reported, studies consistently include sulfathiazole, sulfamethoxazole, and sulfadiazine [3, 7, 18, 23]; others such as sulfadimidine, sulfamoxole, and sulfachloropyrazine are reported only sporadically [15, 24]. Moreover, standards appropriate to our analysis procedure for these compounds were not readily available, and therefore, we decided to include only sulfadiazine, sulfathiazole, sulfamethoxazole, and sulfadoxine in our experiments.

Table 3 shows the results of our analysis of honey purchased in the Czech market. It includes honey from EU countries (*Group 1*), blends of honey from EU and non-EU countries (*Group 2*), and honey from outside the EU (*Group 3*). The values are given as the average of three parallel determinations; "x" indicates that the monitored analyte was not detected. Three honeys containing sulfonamides (highlighted in bold) were found, for which the SDZ (2x) and SMZ (1x) analytes were detected at 232.88, 618.87, and 62.64 μg/kg, respectively, and confirmed according to [12]. Two of these cases were from *Group 1* honeys and were thus in breach of the legislation. In the third case, the sample was from *Group 2*, and it may be assumed that the high level of SDZ residue is possibly due to hive treatment in non-EU countries. However, the "zero tolerance" legislation also applies to this honey with respect to the SAs. Surprisingly, none of the *Group 3* samples contained residual amounts of SAs. This is probably due to the scarce availability of honey only from non-EU countries in the Czech market.

Galarini et al. [16] analyzed 27 antibiotics (sulfonamides, nitroimidazoles, and quinolones) in 74 honeys from the Italian market. In nine honeys (12%), the sulfonamides were found at concentrations 2 μg/kg. This frequency was confirmed by the results of the National Reference Laboratory in Italy, with an 11% incidence of sulfonamides among 1500 honey samples in between 2001 and 2007. The samples included both high and lower quality honeys. In Poland, 20 honeys were examined, and sulfonamides were found in 10 samples (50%) at concentrations 1 to 5.6 μg/kg [25]. Only one honey containing 3.9 μg/kg of sulfamoxole was found by Hu et al. [24] out of 51 samples analyzed. Thirty honeys originating in Greece and imported honey were analyzed by Economou et al. [23]. Of these, four were positive for sulfathiazole (5.3 and 5.9 μg/kg) and sulfamethoxazole (1.5 and 3.4 μg/kg).

Table 1: Retention times, matrix calibration curve, and linear range of the four SAs (LC–MS/MS).

Analytes	Retention time (min)	Calibration curve	Linear range (μg/l)	R^2
SDZ	2.66	$y = 238.125x + 28.0015$	0.1–100	0.997
SDX	3.56	$y = 618.869x + 3.41238$	0.1–100	0.996
SMZ	3.49	$y = 350.874x - 25.7008$	0.1–100	0.996
STZ	2.82	$y = 500.568x - 46.9565$	0.1–100	0.998

SAs: sulfonamides; LC–MS/MS: liquid chromatography with tandem mass spectrometry; R^2: correlation coefficient; SDZ: sulfadiazine; SDX: sulfadoxine; SMZ: sulfamethoxazole; STZ: sulfathiazole.

Table 2: Setting values for mass detection and parameter values multiple reaction monitoring (MRM).

Analyte	Cone (V)	Precursor ion $(M + H)^+$ (m/z)	Product ions (m/z)	Collision (V)	Q (m/z)	C (m/z)
SDZ	34	251	>156	17	92	156
			>92	25		
			>65	39		
SDX	38	311	>156	17	92	108
			>108	30		
			>92	31		
SMZ	37	254	>156	14	92	156
			>108	23		
			>92	27		
STZ	33	256	>156	15	156	92
			>108	24		
			>92	30		

SDZ: sulfadiazine; SDX: sulfadoxine; SMZ: sulfamethoxazole; STZ: sulfathiazole; Q: quantitation fragment; C: confirmation fragment.

Table 3: Results of monitoring study: determination of SAs in honeys from European Union (EU) countries ($n = 25$), from EU and non-EU countries ($n = 20$) and from non-EU countries ($n = 5$) in the Czech market; in units (μg/kg).

	Geographical origin	Botanical origin	SDZ	SDX	SMZ	STZ
EU countries (group 1)						
1	CZ	Blossom	x	x	x	x
2	CZ	Mixed	x	x	**62.64**	x
3	CZ	Honeydew	x	x	x	x
4	CZ	Blossom	x	x	x	x
5	CZ	Blossom	x	x	x	x
6	CZ	Linden	x	x	x	x
7	CZ	Blossom forest	x	x	x	x
8	CZ	Blossom	x	x	x	x
9	SK	Acacia	x	x	x	x
10	CZ	Blossom forest	x	x	x	x
11	CZ	Blossom	x	x	x	x
12	CZ	Blossom	x	x	x	x
13	CZ	Blossom mixed	x	x	x	x
14	CZ	Blossom meadow	x	x	x	x
15	CZ	Linden	x	x	x	x
16	Italy	Honeydew	x	x	x	x
17	Spain	Citrus blossom	x	x	x	x
18	CZ	Blossom	x	x	x	x
19	CZ	Blossom	x	x	x	x
20	CZ	Blossom	x	x	x	x
21	CZ	Blossom	x	x	x	x
22	SK	Blossom	x	x	x	x
23	SK	Blossom	x	x	x	x
24	SK	Blossom	x	x	x	x
25	SK	Blossom	**232.88**	x	x	x

Table 3: Continued.

	Geographical origin	Botanical origin	SDZ	SDX	SMZ	STZ
EU and non-EU countries (group 2)						
26	Not specified	Blossom	x	x	x	x
27	Not specified	Blossom	x	x	x	x
28	Not specified	Blossom	x	x	x	x
29	Not specified	Blossom	x	x	x	x
30	Not specified	Honeydew forest	x	x	x	x
31	Not specified	Blossom	x	x	x	x
32	Not specified	Blossom	x	x	x	x
33	Not specified	Lipový	x	x	x	x
34	Not specified	Blossom honeydew	x	x	x	x
35	Not specified	Blossom	x	x	x	x
36	Not specified	Blossom	x	x	x	x
37	Not specified	Honeydew forest	x	x	x	x
38	Not specified	Blossom	x	x	x	x
39	Not specified	Blossom	x	x	x	x
40	Not specified	Blossom	x	x	x	x
41	Not specified	Meadow	x	x	x	x
42	Not specified	Forest	x	x	x	x
43	Not specified	Honeydew bio	**618.87**	x	x	x
44	Not specified	Blossom bio	x	x	x	x
45	Not specified	Blossom bio	x	x	x	x
Non-EU countries (group 3)						
46	Not specified	Acacia	x	x	x	x
47	Not specified	From mountain flowers	x	x	x	x
48	Not specified	Blossom fruit trees	x	x	x	x
49	Turkey	Honeydew from pines	x	x	x	x
50	Mexico	Blossom	x	x	x	x

SAs: sulfonamides; SDZ: sulfadiazine; SDX: sulfadoxine; SMZ: sulfamethoxazole; STZ: sulfathiazole; x: analyte not found.

As part of the Horizon 2020 program, Juan-Borras et al. [7] analyzed sulfonamides in 279 honeys originating in the Valencia region of Spain. Six samples were positive for sulfathiazole in the range of 5–9 μg/kg and three for sulfadiazine at 13–100 μg/kg. Verzegnassi et al. [18] analyzed honeys originating in Mexico, France, Switzerland, New Zealand, and Vietnam for residues of ten sulfonamides and found sulfamethoxazole at 10.5 μg/kg and sulfathiazole at 114 μg/kg. The values did not correspond to the MRLs adopted by Switzerland (50 μg/kg for total sulfonamides). As part of quality-control monitoring, Vidal et al. [15] analyzed the content of sulfonamides, macrolides, tetracyclines, and quinolones in 16 honeys from Spain (Almeria, Granada) and found residues of sulfadimidine and sulfachloropyridazine. Frerichs et al. [3] investigated German honeys (honey originating in Germany and imported honey) and evaluated the presence of residues of veterinary pharmaceuticals. Their study has highlighted the fact that 50% of the honeys contain at least one antibiotic residue. The analysis was focused on sulfonamides, trimethoprim, and tetracyclines. Imported samples were from Argentina, India, and China. The most common residues were sulfamethoxazole (1316 μg/kg) and trimethoprim (239 μg/kg), which are often used as the cotrimoxazole combination, followed by sulfadiazine, sulfathiazole, sulfamerazine, sulfadimidine, tetracycline, epitetracycline, and oxytetracycline. One honey originating in Hamburg was positive for sulfathiazole at a concentration of 1305 μg/kg.

The results of the pilot experiment for validating the analytical procedure are shown in Tables 1 and 4. Table 1 shows the basic parameters of the matrix calibration curve used to quantify the analytes. These parameters include linearity, the correlation coefficient (>0.996), the retention times, and the equation for the calibration curve for each analyte. Table 4 lists the recovery (R), the intraday-precision (repeatability, RSD_r), and interday-precision (reproducibility, RSD_R)—both expressed as the percentage relative standard deviation. The measurements were carried out at three concentration levels (0.5, 1, and 5 μg/l) with 12 parallel replicates. Recovery values reached a range 60.5–94.6% depending on the analyte and concentration. Repeatability (≤31.6%) was evaluated on the same day, while reproducibility (≤28.8%) was evaluated on three different days. Detection limits (CCα) were 14.4, 27.6, 34.5, and 44.7 μg/kg; detection capabilities (CCβ) were 27.3, 51.0, 68.6, and 91.7 μg/kg for SDX, SMZ, STZ, and SDZ, respectively (Table 4).

These reports make it clear that antibiotics, and particularly sulfonamides, are detected very often in honey. This is despite the ban in EU countries on their use in beekeeping as well as their presence in imported honey. How extrapolations will be taken into account in setting MRLs for honey still remains unresolved [26]. In any case, the principal objective should be to ensure the quality and safety of honey with regard to both hygiene and the health of consumers.

Table 4: Validation parameters of the model study at three concentration levels (n = 12).

Analyte	0.5 μg/l			1.0 μg/l			5.0 μg/l			CCα (μg/kg)	CCβ (μg/kg)
	R (%)	RSD_r (%)	RSD_R (%)	R (%)	RSD_r (%)	RSD_R (%)	R (%)	RSD_r (%)	RSD_R (%)		
SDZ	60.5	31.6	28.8	73.0	30.9	27.4	90.2	14.9	15.6	44.7	91.7
SDX	83.6	24.3	22.4	77.4	22.7	21.6	75.7	13.9	13.2	14.4	27.3
SMZ	94.1	17.4	15.9	82.5	24.0	22.5	76.4	14.3	13.7	27.6	51.0
STZ	94.6	17.9	17.5	79.2	21.4	21.2	66.2	14.0	13.1	34.5	68.6

CCα, decision limit; CCβ, detection capability; R, recovery; RSD_r, intraday-precision (repeatability); RSD_R, interday-precision (reproducibility); SDZ, sulfadiazine; SDX, sulfadoxine; SMZ, sulfamethoxazole; STZ, sulfathiazole.

4. Conclusion

The aim of this study was to evaluate the presence of four sulfonamide residues in fifty honeys sold in the Czech market. Preanalytical preparation was carried out using a modified version of a validated method. Liquid chromatography in conjunction with tandem mass spectrometry was used for the quantification and confirmation required by legislation. Sulfadiazine (232.88 and 618.87 μg/kg) and sulfamethoxazole (62.64 μg/kg) were detected. Positive samples belonged to the group of honey from EU countries (*Group 1*), and blends of honey from EU and non-EU countries (*Group 2*). This is a violation of the ban on the use of sulfonamide pharmaceuticals (with regard to *Group 1*) or inadequate monitoring of the honey trade (with regard to *Group 2*) where sulfonamide therapy is still allowed for the treatment of bee diseases in non-EU countries.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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