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Evaluation of three different microbial inhibition tests for the detection of sulphamethazine residues in the edible tissues of rabbits

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Abstract

The aim of the present study was to evaluate three microbial inhibition tests (MIT) based on the inhibition of the growth of the test organism, (a) the Four Plate Test (FPT) containing *Bacillus subtilis* BGA; (b) the Screening Test for Antibiotic Residues (STAR) containing *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149, and (c) the Premi[®]Test containing *Bacillus stearothermophilus* var. *calidolactis*. The tests were used for determining sulphamethazine (SMZ) residues in edible tissues of rabbits after its oral administration up to day 15 of the withdrawal period (WP). A solvent extraction procedure was used to enhance the capability of the tests to detect SMZ residues at or below maximum residue limit (MRL), and para-aminobenzoic acid (PABA) was employed to identify SMZ residues already in the first stage of the residue screening. The presence of SMZ residues in the samples was confirmed and quantified by a validated HPLC method. The Premi[®]Test detected SMZ residues in the muscle and heart tissue up to day 9 of the WP and in the liver, lungs and kidneys up to day 10 of the WP. The STAR detected SMZ residues in the edible organs of rabbits up to day 8 of the WP. The kidneys were positive up to day 5 of the WP, the liver until day 4 of the WP and the lungs until day 3 of the WP. No SMZ residues were detected in the muscle and heart. By using the solvent extraction procedure, SMZ residues were detected in the muscle extract up to day 10 of the WP and the muscle was positive until day 6 of the WP. No detection sensitivity was observed by using the FPT. After the solvent extraction, SMZ residues were detected in the muscle extract until day 8 of the WP and the muscle was positive until day 3 of the WP. No positive results were detected after the addition of PABA into/onto the agar medium. PABA at the concentration of 10 µg.ml⁻¹ completely reversed the inhibitory activity of SMZ and enabled reliable identification of SMZ in the examined samples. By using the HPLC, SMZ was detected in the muscle samples until day 10 of WP (0.02 mg.kg⁻¹) and in the liver until day 12 of the WP (0.09 mg.kg⁻¹). The results obtained by the HPLC method and the limit of detection (LOD) of screening tests for SMZ (FPT 0.4 µg.ml⁻¹, STAR 0.2 µg.ml⁻¹, Premi[®]Test 0.05 µg.ml⁻¹) allowed us to state that the most suitable screening tests for the detection of SMZ residues in the edible tissues of rabbits at the level corresponding to MRL of 0.1 mg.kg⁻¹, established for sulphonamides, are the Premi[®]Test and the STAR in conjunction with the solvent-extraction procedure.

Keywords: *sulphamethazine, residues, detection, rabbit tissues, microbial inhibition tests, HPLC*

Introduction

Sulphonamides are one of the oldest groups of pharmacologically active substances used in veterinary medicine to date. Their discovery in 1935 signified the beginning of a new era in the treatment of a wide range of bacterial diseases and a number of protozoan infections. In rabbits, sulphonamides have been used for the treatment of coccidiosis. Because of concerns related to sulphonamide residues in food products of animal origin, they must be administered only in recommended concentrations and their respective withdrawal times must be observed..

Council Regulation (EEC) No. 2377/90 establishes the MRL of 0.1 mg.kg^{-1} for sulphonamides (all compounds of the sulphonamide group) in foods of animal origin. The MRL is defined as the maximum concentration of residue resulting from the use of veterinary medicinal products (expressed in mg.kg^{-1} or $\mu\text{g.kg}^{-1}$ on a fresh weight basis), which is legally permitted by the Community as acceptable in or on food (EC 1990). There is a requirement set by the Council Directive 96/23/EC to screen live animals and animal products for residues of antimicrobial drugs (EC 1996).

MIT are widely used in the screening of antimicrobial residues in food-producing animals and their animal products. MIT are based on inhibiting of the growth of test organisms sensitive to many antibiotics and sulphonamides. Among these tests, the FPT (Bogaerts and Wolf 1980), the STAR developed at the Community Reference Laboratory in Fougères (France) and the Premi[®]Test developed by DSM Gist B.V. (The Netherlands) have been most widely used in the residue screening.

The FPT and STAR are plate agar diffusion tests in which the presence of an antibacterial substance is expressed by the production of an inhibition zone. The FPT consists of plates with *Bacillus subtilis* BGA (at pH 6.0, 7.2 and 8.0) and *Micrococcus luteus* ATCC 9341 (at pH 8.0). For the detection of the presence of sulphonamide residues, the FPT prescribes the *Bacillus subtilis* BGA at pH 7.2. The STAR consists of plates with *Bacillus subtilis* BGA (at pH 7.2), *Kocuria varians* ATCC 9341 (at pH 8.0), *Bacillus cereus* ATCC 11778 (at pH 6.0), *Escherichia coli* ATCC 11303 (at pH 8.0) and *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 (at pH 7.4). For the detection of the presence of sulphonamide residues, the STAR prescribes *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149. In order to enhance the sensitivity of the above test organisms to sulphonamides, trimethoprim (TMP) is incorporated in the agar medium at a concentration of $0.05 \mu\text{g.ml}^{-1}$ and $0.005 \mu\text{g.ml}^{-1}$, respectively.

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3 The Premi[®]Test is an agar diffusion test in ampoules in which the presence of an
4 antibacterial substance in concentration exceeding the LOD is indicated by the absence of
5 colour change from purple to yellow of the indicator (bromocresol purple) in the ampoule.
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7 The test organism *Bacillus stearothermophilus* var. *calidolactis* is very sensitive to many
8 antibiotics and sulpha compounds.
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11 An ideal screening method would detect all licensed antimicrobials at or below their
12 MRLs. Both techniques are capable of detecting a range of analytes at or below the MRL
13 concentration. Stead et al. (2004) evaluated an alternative solvent extraction technique for
14 enhancement of the capability of the Premi[®]Test to detect a wider range of drugs at or below
15 $\frac{1}{2}$ MRL in a variety of matrices. They found that the use of the solvent extraction procedure
16 enables effective concentration of the extract to provide increased sensitivity to reach $\frac{1}{2}$ MRL
17 limits that otherwise would not be achievable.
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21 In practice, the samples showing a positive response are subjected to further investigations
22 based on chromatographic methods with specific detectors. Because the final confirmation
23 step can be preceded by preliminary identification and confirmation of sulphonamides by
24 PABA, the post-screening detection of sulphonamides already in the first stage of the residue
25 screening represents an important step of distinguishing the sulphonamides from other classes
26 of veterinary drugs. In the presence of PABA, sulphonamides became weak and lose their
27 antibacterial activity due to antagonistic competition for the synthesis of hydro-folic acid
28 (Gudding 1974; Gudding 1976; Ferrini et al. 1997; Stead et al. 2004; Kožárová et al. 2005;
29 Janošová et al. 2007a; Janošová et al. 2008).
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33 In food analysis, according to the Commission Decision 2002/657/EC, only
34 chromatographic methods can be used for confirmation (EC 2002). The principal approach of
35 the HPLC analysis involves extraction, sample clean-up and HPLC analysis steps. The choice
36 of method used in the laboratory is often dictated by the availability of suitable expertise,
37 facilities, and equipment. However, all analytical methods used in the testing of official
38 samples taken pursuant to Directive 96/23/EC must fulfil criteria set by the Commission
39 Decision 2002/657/EC (EC 2002).
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43 SMZ has been observed to be effective against coccidiosis in rabbits when it is
44 administered orally via drinking water for 3 consecutive days at the dose of 2 g.l⁻¹. To avoid
45 its residues in the edible tissues, a 15-day withdrawal period must be observed before the
46 rabbits are slaughtered for human consumption (Sovík et al. 2005). Because SMZ remains to
47 be the sulphonamide group most widely used in veterinary medicine, the objective of this
48 paper was to determine which of the three aforementioned MIT used in practice today – FPT,
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3 STAR and Premi[®]Test – is most sensitive and suitable for the detection of SMZ residues in
4 the edible tissues of rabbits at the level of concern. To detect the SMZ residues at or below the
5 MRL established, we applied an improved screening technique based on multi-residue solvent
6 extraction. PABA at the concentration of 10 µg.ml⁻¹ was used to confirm the presence of SMZ
7 residues already during the first stage of residue screening. Finally, the level of SMZ residues
8 in the edible tissues of rabbits was determined by validated HPLC analysis.
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12 All four methods mentioned above have been officially approved for screening food-
13 producing animals and their products for residues of veterinary drugs in Slovakia (CH 6.2.
14 2004; CH 12.16. 2004; CH 12.18. 2006; CH 12.19. 2006).
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20 21 **Materials and methods**

22 *Preparation of standard solutions*

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24 The stock solution (1000 µg.ml⁻¹) of the SMZ standard was prepared by dissolving 10 mg of
25 SMZ (Sulfamethazine sodium salt, Sigma S 5637) powder in 2.4 ml ammonia (Lachema,
26 Czech Republic), and diluting it to 10 ml with sterile deionised water. The working solutions
27 of SMZ were prepared by serial dilutions with sterile deionised water to the concentrations of
28 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1 µg.ml⁻¹. Control solutions of SMZ (20 µg.ml⁻¹ for the FPT
29 and 2 µg.ml⁻¹ for the STAR) were used to verify that the operating conditions were
30 systematically respected. The TMP stock solution for the STAR was prepared by dissolving
31 10 mg of TMP standard (Trimethoprim, Fluka 92131) in 1 ml 5% acetic acid (Merck,
32 Germany) and diluting with sterile distilled water to the concentration of 0.5 µg.ml⁻¹. The
33 TMP stock solution for the FPT was prepared by dissolving 10 mg of TMP standard in 10 ml
34 ethanol (Frukona, Slovakia) and diluting it with sterile distilled water to the concentration of
35 5 µg.ml⁻¹. The stock solution of PABA (1000 µg.ml⁻¹) was prepared by dissolving 10 mg
36 PABA (p-aminobenzoic acid, Sigma A 9878) powder in 10 ml distilled water. All stock and
37 working solutions of respective standards were stored in a refrigerator at 4°C.
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51 *Origin and pre-preparation of incurred samples*

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53 Sixteen rabbits (meat line HY+) 16-weeks old, weighing between 2.60 kg–2.90 kg, were
54 obtained for residue analysis from a farm (Hylapa s.r.o., The Slovak Republic). The rabbits
55 were supplied SMZ (Sulfadimidin PG plv. sol. a.u.v., Pharmagal, The Slovak Republic) in
56 drinking water for 3 consecutive days at a dose of 2 g.l⁻¹ of water. After withdrawal of the
57 drug, one rabbit per one day was slaughtered up to day 15 of the WP. The respective samples
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3 of muscle, heart, liver and lung were withdrawn and stored at -20°C until analysis. Six
4 untreated rabbits served as sulphamethazine-free control.
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8 ***Preparation of extract from the rabbit muscle by solvent extraction***

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10 10 g homogenized rabbit muscle was extracted with 25 ml acetonitrile/acetone (70:30, v/v)
11 (Acetonitrile HPLC, Labscan, Ireland; Acetone p.a., Lach-Ners, Czech Republic) on a
12 mechanical shaker LT2 (Votice, Czech Republic) for 15 min. The extract was centrifuged at
13 4500 rpm for 10 min in a centrifuge (Jouan BR 4I, France) at 4°C. The supernatant was
14 removed and evaporated to near dryness under a stream of nitrogen in an evaporator (Turbo-
15 Vap LV, Zymarck, Germany) at 50°C. The residue was suspended in 1 ml Nutrient Agar
16 No. 1 (pH 6.8) (Imuna, The Slovak Republic).
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24 ***Media and strains***

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26 FPT: Test Agar pH 7.2 (Merck, dehydrated medium 15787, Germany), *Bacillus subtilis* BGA
27 (Merck 10649, Germany); STAR: Test agar DST pH 7.4 (Oxoid, dehydrated medium CM
28 261, England), *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 (Merck 1.11499,
29 Germany). Both media were prepared according to the manufacture's instruction.
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33 Premi[®]Test: Premi[®]Test kits, *Bacillus stearothermophilus* var. *calidolactis* (DSM, The
34 Netherlands), ready to use.
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39 ***Preparation of test plates***

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41 FPT: Test Agar pH 7.2 was seeded with the test strain *Bacillus subtilis* BGA to obtain the
42 final concentration of 5×10^4 spores.ml⁻¹ in the agar medium. To obtain the final concentration
43 of TMP in agar medium (0.05 µg.ml⁻¹), 1 ml of TMP solution (5 µg.ml⁻¹) was added to 100 ml
44 of agar medium. STAR: Test agar DST pH 7.4 was seeded with the test strain *Bacillus*
45 *stearothermophilus* var. *calidolactis* ATCC 10149 to give a final concentration of 5×10^6
46 spores.ml⁻¹ in the agar medium. To obtain a final concentration of TMP in agar medium
47 (0.005 µg.ml⁻¹), 1 ml of TMP solution (0.5 µg.ml⁻¹) was added to 100 ml of agar medium.
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49 Finally, 5 ml of the seeded media were transferred into Petri dishes 90 mm in diameter.
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56 ***Determination of LODs***

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58 FPT, STAR: Filter paper discs having a diameter of 12,7 mm (Schleicher & Schull No. 2668,
59 Germany) moistened with 100 µl of respective working solutions of SMZ standard were
60 placed in parallel on the surface of the agar medium in the Petri plates. The plates were

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3 incubated as follows: the plates seeded with *Bacillus subtilis* BGA (FPT) at 30°C for 18–24 h,
4 and the plates seeded with *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149
5 (STAR) at 55°C for 12–15 h.
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9 Premi®Test: 100 µl of respective working solutions of SMZ standard was pipetted onto the
10 agar in the ampoule. Ampoules were incubated in the DSM heating block incubator for
11 approximately 3 h at 64±0.5°C. Each working solution of SMZ standard was tested four-fold.
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15 16 ***Detection of SMZ residues in incurred rabbit samples***

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18 FPT, STAR: A cylindrical core 8 mm in diameter and 2 cm long was removed from each
19 frozen sample using a cork borer. To avoid a doubtful result, each cylindrical core was heat
20 pre-treated in a water bath at 71°C for 20 min. 2 mm slices were cut with a lancet from both
21 end of each core, and were discarded. Eight 2 mm slices were then cut from the remaining
22 rabbit tissue. The slices and the filter paper discs moistened with 100 µl of extract were
23 placed in parallel onto the surface of agar medium in Petri dishes. The plates were incubated
24 as follows: the plates seeded with *Bacillus subtilis* BGA (FPT) at 30°C for 18–24 h, and the
25 plates seeded with *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 (STAR) at
26 55°C for 12–15 h.
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30 Premi®Test: Juice from rabbit tissues obtained by the heat pre-treatment of the samples in a
31 volume of 100 µl was pipetted onto the agar in the ampoule and allowed to stand at room
32 temperature for 20 min for pre-diffusion. After pre-diffusion, the juice was flushed away by
33 washing the test twice with demineralised water. The ampoules were incubated in the DSM
34 heating block incubator for approximately 3 h at 64±0.5°C.
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43 44 ***Presumptive identification of SMZ residues by PABA***

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46 FPT, STAR: The plates used for the confirmatory purposes were prepared by adding 1ml of
47 stock solution of PABA to 100 ml of respective agar medium to obtain the confirmatory
48 PABA concentrations 10 µg.ml⁻¹ in the agar medium.
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51 Premi®Test: 100 µl of stock solution of PABA from the concentration of 100 µg.ml⁻¹ was
52 placed onto the agar in the respective ampoules and allowed to stand at room temperature for
53 20 min for pre-diffusion. After pre-diffusion, the PABA was discharged from the ampoules
54 and both the working solutions of SMZ standard and examined tissue juice were further tested
55 using the Premi®Test procedure mentioned above.
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Reading the test results

FPT, STAR: After incubation, the plates were evaluated and the diameters of clear inhibition zones around the tissue samples and surrounding the filter paper discs moistened with respective solutions of SMZ standard and residue extracts were measured in mm using a digital caliper (Mitutoyo, Japan). The SMZ control disk should present a zone of inhibition of 6 mm according to the FPT and $5\pm 1,5$ mm according to the STAR. The samples are considered positive exhibiting an inhibition zone 2 mm or more according to the FPT and 4 mm or more according to the STAR.

Premi[®]Test: The results were read when the negative control changed colour from purple to yellow. A yellow colour indicated absence of SMZ residues and a purple colour indicated the presence of SMZ residues at or above the LOD of the Premi[®]Test. In the case of doubtful results, the ampoules were incubated a few minutes longer for a clearer result or the samples were retested. In the presence of PABA, the yellow colour indicated that PABA inhibited the antibacterial effect of SMZ and confirmed the presence of SMZ residues. 100 μ l of sterile distilled water was used as a negative control.

Confirmation of the SMZ residues by HPLC

Solvents and reagents

All solvents and reagents were analytical or HPLC grade. Acetonitrile, methanol, n-hexane, and acetic acid were obtained from Merck (Darmstadt, Germany), ethyl acetate, anhydrous sodium sulphate, and sodium acetate from Lachema (Brno, Czech Republic), and deionized and redistilled water was prepared on Milli-Q Plus (Millipore, France).

Sample extraction and sample clean-up

50 g homogenized tissue sample (muscle, liver) was extracted with 50 ml of ethyl acetate on a mechanical shaker for 15 min. The extract was filtered through a filtrate paper with 20 g anhydrous sodium sulphate. The extraction was repeated once more with 50 ml ethyl acetate for 10 min. Both filtrates were collected in a 100 ml round-bottom flask and evaporated to dryness in a rotary vacuum evaporator (Laborota 4000, Heidolph, Merck, Germany) at 40°C. The residue was re-dissolved in 1 ml methanol and 1 ml of mobile phase, and transferred to a centrifuge tube. The flask was washed twice with 2 ml n-hexane, and 4 ml n-hexane was added to the centrifuge tube. The mixture was centrifuged twice at 3500 rpm for 10 min (Jouan BR 41, France). The lower layer was filtered through a disposable syringe filter unit and transferred to the vial. 25 μ l volume of the solution was injected into the HPLC system.

HPLC analysis

Samples were analysed by a TSP liquid chromatograph (Thermo Separation Products, USA) equipped with a variable UV-VIS detector UV 3000 HR (Hewlett-Packard, USA). The separation was performed on a LiChroCART RP-18e column (125–4, 5 μm) (Merck, Germany) using an acetonitrile-acetate buffer (pH 4,6) (25:75, v/v) as a mobile phase at a flow-rate of 0,8 $\text{ml}\cdot\text{min}^{-1}$ at the temperature of 40°C. The detection was conducted at 275 nm.

Results

The initial experiment was undertaken to detect the LOD of the screening methods for SMZ by determining the MIC of the SMZ standard for the respective test strains of screening methods. Because the MIC is defined as the lowest concentration of the respective antibiotic standard which completely inhibits the growth of the sensitive test organism, the diameters of the inhibition zones (FPT, STAR) and the colour change which represented the positive or negative results (Premi[®]Test) were evaluated. In an attempt to confirm the presence of SMZ residues already during the first stage of residue screening, PABA at the concentration of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ was used. This PABA concentration was taken into consideration according to the results obtained in our preliminary studies (Kožárová et al. 2005; Janošová et al. 2007a). All the results are summarized in Table I. Our results showed that the MIC of SMZ was 0.4 $\mu\text{g}\cdot\text{ml}^{-1}$ for FPT, 0.2 $\mu\text{g}\cdot\text{ml}^{-1}$ for STAR, and 0.05 $\mu\text{g}\cdot\text{ml}^{-1}$ for Premi[®]Test /A/. By adding PABA to the agar medium (FPT, STAR) or onto the top of the agar medium (Premi[®]Test), the absence of the inhibition zones or yellow colour was observed /B/.

The results of the screening for the presence of SMZ residues in the edible tissues of rabbits after its oral administration up to day 15 of the withdrawal period are presented in Tables II, III, and IV. Tables II and III show the mean diameters of the inhibition zones ($\text{mm}\pm\text{SD}$ /standard deviation/) produced by SMZ residues around the examined rabbit tissues and discs moistened with the extract from the rabbit muscle. Table IV shows the sensitivity of the test towards SMZ residues presented in the juice from rabbit tissue. By adding PABA to the agar medium (FPT, STAR) or onto the top of the agar medium (Premi[®]Test), no positive results were detected. PABA at the concentration of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ completely reversed the inhibitory activity of SMZ and identified reliably the presence of SMZ in the examined samples. Because all results with plates B or ampoules B were identical (0 /FPT, STAR/, – /Premi[®]Test/), Tables II, III, and IV contain the results observed at the screening of SMZ residues in edible tissues of rabbits only with plates A.

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3 No detection sensitivity presented by the absence of the inhibition zones around the
4 examined rabbit tissues was observed when using the FPT (Table II. /A/). When using the
5 solvent extraction, the FPT detected the presence of SMZ residues in examined rabbit muscle
6 tissue until day 8 of the WP. Because the FPT designated the sample positive at the inhibition
7 zone of 2 mm and more, the muscle was positive until day 3 of the WP, and the mean
8 diameter of the inhibition zone was 2.83 ± 0.13 mm. According to the results obtained by
9 screening of the SMZ standard, the residual concentration of SMZ in the positive muscle
10 tissue was $0.4\text{--}0.5 \mu\text{g}\cdot\text{g}^{-1}$.
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17 The STAR detected SMZ residues in the examined rabbit tissues (lungs, liver and
18 kidneys) until day 8 of the WP. No SMZ residues were detected in the muscle and heart
19 (Table III. /A/). Because the STAR rated the sample positive if the inhibition zone was 4 mm
20 and greater, the liver was positive until day 4 of the WP (mean inhibition zone 4.21 ± 0.08
21 mm), the lungs were positive until day 3 of the WP (mean inhibition zone 4.01 ± 0.48 mm), and
22 the kidneys were positive until day 5 of the WP (mean inhibition zone 4.35 ± 0.25 mm).
23 According to the results obtained by screening of the SMZ standard, the residual
24 concentration of SMZ in the presented positive rabbit tissue was in the range of $0.4\text{--}0.5 \mu\text{g}\cdot\text{g}^{-1}$.
25 After isolation of SMZ residues from the muscle by solvent extraction, the SMZ residues
26 were detected in the muscle extract until day 10 of the WP and the muscle was positive until
27 day 6 of the WP (mean inhibition zone 4.32 ± 0.19). According to the results obtained by
28 screening of the SMZ standard, the residual concentration of SMZ in the presented positive
29 rabbit muscle tissue was $0.4\text{--}0.5 \mu\text{g}\cdot\text{g}^{-1}$.
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40 The Premi[®]Test detected SMZ residues in all examined matrices. By using the fluid
41 expression procedure, the muscle and heart were positive until day 9 of the WP, and the liver,
42 lungs and kidneys until day 10 of the WP (Table IV /A/). The positive results manifested by
43 the maintenance of the violet colour of the agar medium imply the presence of SMZ residues
44 at or above the LOD of the method. The negative results manifested by decolouration of the
45 agar medium imply the absence of SMZ or presence of its residues below the LOD of the
46 method. According to the results obtained by screening of the SMZ standard we can assume
47 that the residual concentration of SMZ in the presented positive rabbit tissues was $0.05 \mu\text{g}\cdot\text{ml}^{-1}$.
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56 No positive results implied by the presence of inhibition zones or by maintenance of
57 violet colour of the agar medium were observed in all examined rabbit tissues obtained from
58 SMZ-free control group.
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HPLC analysis

The presence of SMZ residues in the rabbit tissues (muscle, liver) throughout the 15 days of the WP were consequently confirmed and quantified by HPLC. The LOD of the method for SMZ was 0.004 mg.kg^{-1} and the respective limit of quantification (LOQ) for SMZ was 0.010 mg.kg^{-1} . Recoveries of SMZ from spiked control sample at the concentration from 0.3 mg.kg^{-1} to 3 mg.kg^{-1} ranged between 83% and 88%. SMZ residues were detected with UV-VIS detector at 275 nm at the retention time between 6.958 and 7.731 min. The mean residue concentrations of SMZ (mg.kg^{-1}) in the examined samples are shown in Table V.

Table V shows that SMZ residues were detected in both examined matrices and the decrease in SMZ level was gradual and very slow. The highest residue concentrations were found on the first day of the WP. On this day, the mean residue concentration of SMZ in the liver was 0.56 mg.kg^{-1} , and in the muscle it was 0.42 mg.kg^{-1} . SMZ residues in the liver of rabbits were detectable up to day 12 of the WP and the mean SMZ residue concentration was 0.09 mg.kg^{-1} . On day 13 of the WP, SMZ residues were below the LOD of the method. SMZ residues in the rabbit muscles were detectable up to day 10 of the WP and the mean SMZ residue concentration was 0.02 mg.kg^{-1} . On the day 11 of the WP, SMZ residues were below the LOD of the method. No residues of SMZ were found in the muscles and liver obtained from the SMZ-free control group.

Discussion

The presence of contaminants and veterinary drug residues in products of animal origin is a matter of concern for public health (Danko et al. 2005; Koréneková et al. 2007; Lukáč and Massányi 2007). The strategy of official residue control is based on two sequential steps: screening and confirmation. According to the Commission Decision 2002/657/EC a screening method means method that is used to detect the presence of a substance or class of substances at the level of interest. Screening methods have the capability for a high sample throughput and are used to sift large number of samples for potential non-compliant results. They are specifically designed to avoid false compliant results. Confirmatory method means method that provides full or complementary information enabling the substance to be unequivocally identified and, if necessary, quantified at the level of interest. The level of interest means the concentration of substance or analyte in a sample that is significant for determination of its compliance with legislation (EC 2002).

Live animals and animal products are often screened for antibiotic and sulphonamide residues in the first stage of residue screening by using the microbial inhibition tests. There is

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2
3 a whole range of such tests with various test organisms used worldwide. The LOD of the MIT
4 for a given antibacterial depends primarily on the innate sensitivity of the test organism. In
5 sulphonamide residue screening, four test organisms, namely *Bacillus subtilis* BGA, *Bacillus*
6 *megaterium* ATCC 9885, *Bacillus stearothermophilus* var. *calidolactis*, and *Bacillus pumilus*
7 have been most widely used and investigated. The published data show that their sensitivity is
8 not equal for all compounds of the sulphonamide group. Their sensitivity to sulphonamides
9 were often affected by differences in test media, depth of agar layers, pH value of the agar
10 medium, addition of TMP or dextrose to the agar medium, temperature, preparation and
11 application of the standard solution, animal tissues, etc. (Bogaerts and Wolf 1980; Hrdlička
12 1990; Heitzman 1994; Koenen-Dierick and De Beer 1998; Okerman and Van Hoof 1998;
13 Okerman et al. 1998; Korsrud et al. 1998; Hussein 2004; Gaudin et al. 2004; Dey et al. 2005;
14 Kožárová and Labanská 2005; Janošová et al. 2007b; Pikkemaat et al. 2008).

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25 PABA specifically inhibits the bacteriostatic activity of sulphonamides. Many authors
26 investigated PABA at its various concentrations (from 0.1 $\mu\text{g}\cdot\text{ml}^{-1}$ to 100 $\mu\text{g}\cdot\text{ml}^{-1}$) for
27 identification of sulphonamide residues at the first stage of their residue screening (Lin et al.
28 1995; Ferrini et al. 1997; Braham et al. 2001; Stead et al. 2004; Kožárová et al. 2005,
29 Janošová et al. 2007a, Janošová et al. 2008). All authors found that in the absence of PABA,
30 sulphonamides produced inhibition zones on the test plates, whereas in its presence, no
31 inhibition zones or reduction of their diameters were observed. The confirmatory PABA
32 concentration was often conditioned by respective TMP concentration in the agar medium.

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Chemical denaturation of tissues assists with release of drug residues. Stead et al. (2004)
improved screening technique for rapid detection of antimicrobial residues in animal food
products using of a solvent extraction procedure. The use of the solvent extraction technique
coupled to the Premi[®]Test provides an enhanced test capable of detecting a wider range of
drugs at or below maximum residue limit concentrations. A solvent ratio of 70:30 v/v
acetonitrile/acetone was found experimentally to be optimum for extracting the widest range
of analytes including sulphonamides.

The samples showing positive responses are subjected to further investigations by
quantitative physico-chemical analysis. Chromatographic methods are being developed to
confirm the findings of the screening methods. To determine sulphonamide residues in
different matrices, several chromatographic methods based on HPLC with ultraviolet,
fluorescence or mass spectrometry detection have been used in practise.

Bearing in mind these consideration, the aim of our study was to evaluate three MIT used
in practise today, the FPT containing *Bacillus subtilis* BGA, the STAR containing *Bacillus*

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3 *stearothermophilus* var. *calidolactis* ATCC 10149, and the Premi[®]Test containing *Bacillus*
4 *stearothermophilus* var. *calidolactis*, for determining SMZ residues in the edible tissues of
5 rabbits after its oral administration up to day 15 of the withdrawal period at the level of
6 concern. To obtain reliable results already in the first stage of residue screening and to
7 identify the presence of SMZ residues in the examined rabbit samples, both the screening
8 procedure based on solvent extraction step and the post-screening identification of SMZ by
9 PABA were used. The confirmatory analysis was undertaken by validated HPLC method.

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16 The LODs of all tested MIT for SMZ were determined by detecting the MIC of SMZ
17 standard which inhibited the growth and multiplication of the test organism with the
18 respective method. The test organism *Bacillus stearothermophilus* var. *calidolactis* showed
19 higher sensitivity to SMZ than the test organism *Bacillus subtilis* BGA. According to our
20 results, the LOD of MIT for SMZ were as follows: FPT 0.4 $\mu\text{g}\cdot\text{ml}^{-1}$, STAR 0.2 $\mu\text{g}\cdot\text{ml}^{-1}$, and
21 Premi[®]Test 0.05 $\mu\text{g}\cdot\text{ml}^{-1}$. The Premi[®]Test detected SMZ residues (positive results) in the
22 muscles and heart tissues until day 9 of the WP, and in the liver, lungs and kidneys until day
23 10 of the WP. The STAR detected SMZ residues in the edible organs of rabbits until day 8 of
24 the WP. The kidneys were positive until day 5 of the WP, the liver until day 4 of the WP, and
25 the lungs until day 3 of the WP. No SMZ residues were detected in the muscle and heart. The
26 FPT detected no SMZ residues in any examined matrices.

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35 The use of the solvent extraction significantly improved the existing screening
36 methodology of plate agar diffusion methods and confirmed the data presented by the author
37 mentioned above. Mixture of acetonitrile/acetone at a solvent ratio of 70:30 v/v, enabled
38 extraction of SMZ residue from “negative” muscle tissues and enhanced the capability of the
39 STAR and the FPT for detecting SMZ residues at the level of interest. By using the STAR,
40 SMZ residues were detected in the muscle extract until day 10 of the WP. The muscles were
41 positive until day 6 of the WP. By using the FPT, SMZ residues were detected in the muscle
42 extract until day 8 of the WP and the muscles were positive until day 3 of the WP.

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49 PABA, at the concentration of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ completely inhibited the antibacterial effect of
50 SMZ in all examined matrices. The absence of inhibition zones around the examined matrices
51 on PABA containing agar and visible decolourization of the agar medium from purple to
52 yellow after adding PABA to the ampoule indicated the presence of SMZ residues in the
53 samples. These results confirmed that PABA is an efficient and reliable agent for presumptive
54 identification of SMZ residues in screen-positive samples.

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60 The choice of method for confirmatory residue analysis is often dictated by the
availability of suitable facilities and equipment. The HPLC method used in our study was

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3 found to be suitable for reliable detection of SMZ residues in the edible tissues of rabbits
4 bellow the MRL level of 0.1 mg.kg⁻¹. The LOD and LOQ of the method for SMZ were 0.004
5 mg.kg⁻¹ and 0.010 mg.kg⁻¹, respectively. The SMZ was detected in the muscle samples until
6 day 10 of WP at the residual concentration of 0.02 mg.kg⁻¹, and in the liver until day 12 of the
7 WP at the residual concentration of 0.09 mg.kg⁻¹.

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12 With regard to the results obtained by the HPLC method we can conclude that the most
13 suitable MIT for the detection of SMZ residues in the edible tissues of rabbits at the level of
14 interest is the Premi[®]Test followed by the STAR coupled with the solvent extraction
15 procedure. The solvent extraction step markedly influenced the results of both plate agar
16 diffusion methods, the STAR and the FPT, and positively improved their detection capability.
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22 23 24 25 **Conclusions**

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27 The residue-monitoring process is critical to the protection of the consumer's health. It must
28 be effective and match the patterns of use for all classes of drugs applied in animal-production
29 system, including sulphonamides. MIT are highly valuable in the first stage of residue
30 screening. The purpose of these screening methods is to give a quick result whether the
31 residue is either not present in the sample or is at or bellow the level of interest. With regard
32 to their excellent practicality and throughput, they allow us to classify a large number of
33 samples as being either "negative" or "potentially positive". All potentially positive samples
34 are then subjected to a confirmatory test.
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42 Because SMZ is still widely used for the treatment of coccidiosis in all food-producing
43 animals, SMZ residues remain an important consideration for consumers. Thus the objective
44 of the present work was to detect which of the three aforementioned MIT, used in practise
45 today, the FPT, the STAR and the Premi[®]Test, is most suitable for relevant detection of SMZ
46 residue in the edible tissues of rabbits without any risk for consumers. Our results showed that
47 the commercially produced Premi[®]Test is by far the most suitable MIT for the detection of
48 SMZ residues bellow the level of the MRL 0.1 mg.kg⁻¹. The STAR detected SMZ residues
49 closely above the MRL level, and the FPT above the MRL level. This indicates that the MIT
50 containing *Bacillus stearothermophilus* var. *calidolactis* the test organism are more sensitive
51 to SMZ than those with *Bacillus subtilis* BGA. When the edible tissues of rabbits were
52 screened by classical methodologies of the STAR and the FPT, no SMZ residues were
53 detected in the rabbit muscles. The alternative screening technique based on solvent extraction
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3 permitted apparent enhancement of the test detection capability and improved positive results
4 of both less sensitive MIT. Because the MIT are sensitive to a wide range of antimicrobial
5 compounds, the use of PABA in post-screening investigation of the screen-positive samples
6 allowed us to identify the presence of SMZ residues in the examined samples and choose the
7 method suitable for confirmatory analysis. HPLC analysis of muscle and liver samples of
8 rabbits confirmed the presence of SMZ residues in both examined matrices.
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14 The results obtained allowed us to conclude that the Premi[®]Test and the STAR with the
15 test organism *Bacillus stearothermophilus* var. *calidolactis* ATCC 10149 in combination with
16 the alternative screening technique based on the solvent extraction procedure, can be used as
17 the most suitable screening tests for detection of SMZ residues in the edible tissues of rabbits
18 at the level corresponding to MRL of 0.1 mg.kg⁻¹. The utilisation of PABA for the
19 presumptive identification of SMZ residues in the screen-positive samples constitutes an
20 impressive challenge to employ efficient and cost-effective means of controlling SMZ
21 residues in foods.
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30 **Acknowledgements**

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Table I. LOD of screening methods for SMZ determined by the MIC of SMZ standard presented as the lowest mean diameter of the inhibition zones (mm \pm SD) or positive results (A) and presumptive identification of SMZ with PABA at the concentration of 10 $\mu\text{g}\cdot\text{ml}^{-1}$ (B).

MIT	Plate/ Ampoule	Concentration of SMZ ($\mu\text{g}\cdot\text{ml}^{-1}$)						
		0.05	0.1	0.2	0.3	0.4	0.5	1
FPT	A	0	0	0	0	2.62 \pm 0.26	3.09 \pm 0.17	3.67 \pm 0.14
	B	0	0	0	0	0	0	0
STAR	A	0	0	1.09 \pm 0.48	2.17 \pm 0.75	3.17 \pm 1.17	4.00 \pm 0.63	5.00 \pm 0.63
	B	0	0	0	0	0	0	0
Premi [®] Test	A	+	+	+	+	+	+	+
	B	-	-	-	-	-	-	-

Bold concentrations represent the LOD of respective MIT for SMZ; + positive results; All data are the mean \pm SD of six observations.

Table II. The mean diameters of the inhibition zones (mm \pm SD) observed at the screening of SMZ residues in edible tissues of rabbits after its oral administration up to the day 15 of the withdrawal period using the FPT with *Bacillus subtilis* BGA at pH 7.2.

Day of WP	Plate	Muscle	Muscle extract	Heart	Liver	Lungs	Kidneys
1.	A	0	8.04 \pm 1.07	0	0	0	0
2.	A	0	4.79 \pm 0.35	0	0	0	0
3.	A	0	2.83 \pm 0.13	0	0	0	0
4.	A	0	1.73 \pm 0.26	0	0	0	0
5.	A	0	1.72 \pm 0.15	0	0	0	0
6.	A	0	1.64 \pm 0.12	0	0	0	0
7.	A	0	1.46 \pm 0.23	0	0	0	0
8.	A	0	0.75 \pm 0.60	0	0	0	0
9.–15.	A	0	0	0	0	0	0
Control sample	A	0	0	0	0	0	0

A – plates without the addition of PABA into the agar medium; Bold numerals represent the positive results; All data are the mean \pm SD of four observations.

Table III. The mean diameters of the inhibition zones (mm \pm SD) observed at the screening of SMZ residues in edible tissues of rabbits after its oral administration up to the day 15 of the withdrawal period using the STAR with *Bacillus stearothermophilus* ATCC 10149 at pH 7.4.

Day of WP	Plate	Muscle	Muscle extract	Heart	Liver	Lungs	Kidneys
1.	A	0	9.91 \pm 0.55	0	8.12 \pm 1.78	5.85 \pm 0.67	8.75 \pm 0.39
2.	A	0	8.16 \pm 1.04	0	5.39 \pm 0.67	4.17 \pm 0.63	6.53 \pm 0.20
3.	A	0	7.93 \pm 1.01	0	4.46 \pm 0.53	4.01 \pm 0.48	5.59 \pm 1.30
4.	A	0	6.65 \pm 1.39	0	4.21 \pm 0.08	2.42 \pm 0.49	4.45 \pm 0.71
5.	A	0	5.01 \pm 0.57	0	2.54 \pm 0.66	1.,28 \pm 0.25	4.35 \pm 0.25
6.	A	0	4.32 \pm 0.19	0	2.01 \pm 0.51	1.08 \pm 0.23	3.05 \pm 0.26
7.	A	0	2.36 \pm 0.38	0	1.66 \pm 0.13	0.71 \pm 0.84	2.53 \pm 0.19
8.	A	0	2.31 \pm 0.28	0	1.57 \pm 1.08	0.63 \pm 0.76	1.95 \pm 0.27
9.	A	0	2.26 \pm 0.90	0	0	0	0
10.	A	0	1.29 \pm 0.51	0	0	0	0
11.–15.	A	0	0	0	0	0	0
Control sample	A	0	0	0	0	0	0

A – plates without the addition of PABA into the agar medium; Bold numerals represent the positive results; All data are the mean \pm SD of four observations.

Table IV. Positive and negative results observed at the screening of SMZ residues in edible tissues of rabbits after its oral administration up to the day 15 of the withdrawal period using the Premi[®]Test.

Day of WP	Ampoule	Muscle	Heart	Liver	Lungs	Kidneys
1.	A	+	+	+	+	+
2.	A	+	+	+	+	+
3.	A	+	+	+	+	+
4.	A	+	+	+	+	+
5.	A	+	+	+	+	+
6.	A	+	+	+	+	+
7.	A	+	+	+	+	+
8.	A	+	+	+	+	+
9.	A	+	+	+	+	+
10.	A	-	-	+	+	+
11.-15.	A	-	-	-	-	-
Control sample	A	-	-	-	-	-

A – ampoule without the addition of PABA onto the agar medium; + positive results; – negative results; All data are the mean \pm SD of four observations.

Table V. SMZ-residue concentrations ($\text{mg.kg}^{-1} \pm \text{SD}$) in the rabbit muscle and liver after its oral administration up to the day 15 of the withdrawal period detected by the HPLC.

Day of WP	Muscle	Liver
1.	0.42 ± 0.046	0.56 ± 0.021
2.	0.38 ± 0.011	0.49 ± 0.032
3.	0.33 ± 0.033	0.41 ± 0.017
4.	0.33 ± 0.015	0.39 ± 0.009
5.	0.27 ± 0.007	0.39 ± 0.011
6.	0.25 ± 0.004	0.31 ± 0.013
7.	0.21 ± 0.025	0.27 ± 0.015
8.	0.18 ± 0.016	0.25 ± 0.008
9.	0.15 ± 0.002	0.21 ± 0.050
10.	0.02 ± 0.004	0.21 ± 0.070
11.	ND	0.19 ± 0.006
12.	ND	0.09 ± 0.003
13.–15.	ND	ND

Bold numerals represent the days of the WP when the lowest residue levels were measured; ND – not detectable.