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A NOVEL APPROACH TO CONTROL SULFAMETHAZINE MISUSE IN FOOD PRODUCING ANIMALS BY HAIR ANALYSIS

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4 1 **A novel approach to control sulfamethazine misuse in food**
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6 2 **producing animals by hair analysis**
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11 4 Marta Gratacós-Cubarsí, Massimo Castellari*, Angel Valero, Isabel Diaz, José
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15 **Abstract**

16 The presence of sulfamethazine (SMZ) residues in pig and calf hair was
17 compared with the residual levels encountered in the corresponding edible
18 tissues (liver and muscle) as a consequence of drug administration. SMZ up to
19 84.7 mg/kg was found in calf hair samples after a pharmacological treatment,
20 with a significant effect on hair pigmentation. High concentrations of the parent
21 drug were detected in calf hair for four weeks after administration, when SMZ
22 residues were no longer detectable in the corresponding edible tissues. In a
23 similar way, pig hair also accumulated SMZ residues up to 40.5 mg/kg, which
24 was more than the amount detected in the corresponding muscle and liver
25 samples at slaughter. Hair analysis seems a suitable tool to improve the
26 efficacy of regulatory controls, and thus the safety of the food chain and to
27 discourage the improper use of SMZ in animal farming.

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31 Introduction

32 Sulfonamides (SAs) are an important group of synthetic bacteriostatics which
33 are approved as therapeutic agents for use on farm. The sub-therapeutic
34 addition to feeding to improve growth promotion and food efficiency is permitted
35 in the USA (Animal Health Institute) but is prohibited within the European Union
36 (EC 2003). Several years ago the scientific community was alerted to the
37 excessive use of SAs (and other antimicrobials) which can promote the
38 development of microbial resistance (WHO 1997, WHO 1998, Cruz et al., 2003;
39 Pena et al., 2004; Miko et al., 2005) as well as a progressive environmental
40 contamination (Verstraete 2004; Kay 2005a; 2005b). Significantly,
41 sulfamethazine (SMZ), one of the most used SAs, has also been proved to
42 induce tumors in the thyroid gland of rodents (Littlefield et al., 1989).

43
44 At the same time, the illegal or improper administration of SAs, such as the non-
45 observance of withdrawal time or the use without veterinary prescription, may
46 cause residues in the edible tissues of slaughtered animals that could represent
47 a potential risk to consumer health due to the short-term toxic and allergic
48 phenomena in sensitized consumers (European Medicines Agency).

49
50 Following the rules established for other veterinary drug, a Maximum Residue
51 Level (MRL), which has also been called Safe Level or Tolerance Limit, has
52 been defined for SAs in edible animal tissues or other foodstuffs of animal origin
53 in several countries (Codex Alimentarius, EC 1990, Code of Federal
54 Regulation). The MRL represents a drug residual level which is virtually
55 associated with no toxicological risk for the consumer, and is calculated taking

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3 56 into account toxicity studies, food daily intake as well as other safety factors.

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5
6 57 The MRL for the SMZ is set at 100 $\mu\text{g}/\text{Kg}$ for all the animal species and tissues

7
8 58 (EC 1990).

9
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12
13 60 The official target samples (meat, kidney, fat and liver) are usually taken at the

14
15 61 time of slaughter to verify the compliance with withdrawal time and MRL.

16
17 62 Nevertheless the *post-mortem* sampling of edible tissues can only be made on

18
19 63 a limited percentage of carcasses and may delay the commercialization of the

20
21 64 edible parts. Furthermore the half-life of drug residues in other samples taken

22
23 65 from living animals (milk, urine, faeces) is generally shorter compared to the

24
25 66 withdrawal period for edible tissues (USP DI), limiting the efficacy of these

26
27 67 matrices for screening controls at farm and at slaughter.

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33 69 Recently, hair analysis has been proposed as an interesting technique to

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35 70 increase the time window in retrospective detection of veterinary drug residues,

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37 71 offering at the same time the advantage of non-invasive collection on living

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39 72 animals (Durant et al., 2002; Nielen et al., 2003; Dunnett and Lees 2004a;

40
41 73 2005b). It is well known that hair structure is rather biologically inert allowing this

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43 74 to produce a protected microenvironment where the drug residue would be

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45 75 preserved from degrading agents during a long period of time (Dunnett and

46
47 76 Lees, 2004a; 2004b).

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53 78 At present, hair analysis is considered an effective tool to detect banned drugs

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55 79 as anabolic hormones (Gratacós-Cubarsí et al., 2006c), but it seems also

56
57 80 interesting to control the administration of compounds authorized only for

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3 81 therapeutic uses (corticosteroids, antibiotics) which should be dispensed under
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5 82 strict veterinary prescription.
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9
10 84 It has been established that SAs are deposited in the hair structure after oral
11
12 85 administration or intravenous injection in racehorses (Dunnett and Lees,
13
14 86 2004b), calf and pig (Gratacós-Cubarsi et al., 2006a) hairs. SAs residues at
15
16 87 mg/kg level have been detected in hair several months after administration, and
17
18 88 a relationship between administered dose and residue level in hair was
19
20 89 established for horsehair (Dunnett and Lees, 2004b). To the best of our
21
22 90 knowledge, no bibliographic data is available to compare the residue levels in
23
24 91 hair and other biological samples of the same animal after SA treatment.
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31 93 The primary objective of the work reported here was the comparison of the
32
33 94 residual SMZ levels in edible tissues (liver and muscle) and hair of pigs and
34
35 95 calves which received a pharmacological treatment during the rearing. The
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37 96 effects of several factors (hair pigmentation, individual response, time between
38
39 97 administration and sampling) on SMZ accumulation in hair samples were also
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41 98 determined, to assess the suitability of hair analysis as a complementary tool in
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43 99 official residues monitoring plans.
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50 101 **Methods and Procedures**

52 102 *Reagents*

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54 103 Sulfamethazine (SMZ, min 99 %) [4-amino-N-(4,6-dimethyl-2-pyrimidinyl)-
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56 104 benzenesulfonamide] and ammonium hydroxide ACS reagents were purchased
57
58 105 from Sigma-Aldrich® (Madrid, Spain). Organic solvents (methanol and
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60

1
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3 106 acetonitrile liquid chromatography grade), ammonium acetate and acetic acid
4
5 107 glacial were from Merck (Darmstadt, Germany). Ultra-pure water was obtained
6
7
8 108 using an E-pure system (Barnstead Thermolyne, Dubuque IA-USA).

9
10 109 Two different veterinary drugs were employed for the pharmacological
11
12 110 treatments, an injectable solution (Copisulf, containing 330 mg/mL
13
14 111 sulfamethazine sodium salt, from FEDRA S.A., Girona, Spain) and a soluble
15
16 112 powder (Torul S, containing 550 mg/g SMZ sodium salt, from Lamons,
17
18 113 Tarragona, Spain).

19
20 114 A standard stock solution [1 g/L] of SMZ was prepared by dissolving 100 mg of
21
22 115 pure standard in 100 mL of methanol. The standard working solution [1 mg/L]
23
24 116 was prepared weekly by dilution of standard stock solution in ammonium
25
26 117 acetate 0,01M pH 4.5: acetonitrile (85:15 v/v) and stored at 4 °C.

27
28 118 SPE HLB-OASIS[®] (6cc/200 mg) cartridges were obtained from Waters Corp.
29
30 119 (Barcelona, Spain) while strong cationic exchange cartridges (Accubond^{II} SCX,
31
32 120 6cc/500 mg) were acquired from Agilent Technologies S.L. (Madrid, Spain).

33 121 *Animal treatment*

34 122 Six Friesian calves were reared in our experimental farm to 8 months receiving
35
36 123 no medication containing sulfonamides. At five weeks before slaughter they
37
38 124 were divided into three groups, and kept in separate stalls during the last
39
40 125 growing phase.

41
42 126 The first group (Control) did not receive any veterinary drug. The second group
43
44 127 (WP, see Figure 1), complying with a withdrawal period of 28 days, was given a
45
46 128 daily subcutaneous dose of Copisulf (40 mL) per animal for 5 days
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48 129 (corresponding to 150-165 mg sulfamethazine sodium salt/kg live weight).

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3 130 A third group received the same treatment with Copisulf just one week before
4
5 131 slaughter without having the withdrawal period (NoWP, see Figure 1). Treated
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8 132 animals received the daily dose in different zones of the thorax to avoid the
9
10 133 development of epithelial lesions.

11
12 134 Six Landrace pigs were also reared in the same experimental farm to 3 months
13
14
15 135 receiving no drugs containing sulfonamides. Four weeks before slaughter, a
16
17 136 group of three animals (NoWP, see Figure 1) started receiving a daily oral dose
18
19 137 of 10 g of Torul S mixed with feeding for 28 days (corresponding to a SMZ dose
20
21 138 of 1.40 - 1.54 g sulfamethazine sodium salt/Kg live weight). The other group of
22
23 139 three animals received no medication (Control).

24 25 26 27 140 *Sampling*

28
29 141 Pigmented and non-pigmented hair samples were taken (2.0-3.0 g. approx.
30
31 142 each) from both treated and control animals before and after the beginning of
32
33 143 the pharmacological treatments with SMZ (Figure 1).

34
35 144 Sequential samples of the same animal were taken from different zones of the
36
37 145 upper area of the neck (to limit external contamination) using a hair clipper
38
39 146 ARCO 1854 (MOSER, Germany) with a cut height of 1 mm.

40
41 147 In the case of the calves having a withdrawal period (WP), additional samples
42
43 148 were taken from the areas of the first sampling, corresponding to the hair shaft
44
45 149 grown during the two weeks after the first sampling (NG-1) or the last three
46
47 150 weeks before slaughter (NG-2) (Figure 1).

48
49 151 At slaughter samples of muscle and liver were taken from both Control and
50
51 152 treated animals. These tissues were immediately stored at -20 °C and analyzed
52
53 153 within 3 months.

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5 155 *SMZ extraction*

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8 156 Hair samples were washed three times using ultra-pure water, dried in an oven
9
10 157 at 37 °C for one hour and then analyzed by HPLC-DAD to quantify the SMZ
11
12 158 content as previously described (Gratacós-Cubarsí et al., 2006a).

13
14
15 159 Briefly, 50 mg of finely cut calf hair or pulverized pig hair were extracted with 4
16
17 160 ml of aqueous alkaline solution. Crude alkaline extracts were centrifuged and
18
19 161 filtered through a paper filter. Centrifuged alkaline extracts were directly loaded
20
21 162 onto an OASIS[®]-HLB cartridge, washed with 2 mL of 5 % methanol in water.
22
23 163 SMZ was eluted with 4 mL acetic acid: acetonitrile (5:95 v/v), and directly
24
25 164 loaded onto the SCX cartridge. After washing with 5 mL methanol:acetonitrile
26
27 165 (1:1 v/v), SMZ was eluted with 5 mL of ammonium hydroxide: methanol (5:95
28
29 166 v/v).

30
31
32 167 Eluates containing SMZ were evaporated under nitrogen at 25 °C, reconstituted
33
34 168 with 400 µL of ammonium acetate 0,01M pH 4.5: acetonitrile (88:12 v/v) and
35
36 169 filtered through a 0.45 µm nylon filter.

37
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39 170 To evaluate the SMZ residues in muscle and liver, 4 g of sample were extracted
40
41 171 with 20 mL of acetonitrile and centrifuged. One mL of the supernatant was
42
43 172 evaporated with nitrogen, reconstituted with 0.5 mL of the mobile phase, filtered
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45 173 with 0.45 µm nylon membranes and analyzed by HPLC (Gratacós-Cubarsi et
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47 174 al., 2006b).

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55 176 *HPLC conditions*

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3 177 Chromatographic separations were carried out on an 1100 liquid
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5 178 chromatograph (Agilent Technologies, Palo Alto, CA - US) equipped with a
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8 179 quaternary pump and a diode array detector.

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10 180 The analysis of hair extracts were carried out with a Luna[®] RP-C₁₈ (150 mm x
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12 181 2.0 mm i.d.) analytical column (Phenomenex[®], Torrance - CA, US). A binary
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14
15 182 gradient elution (flow rate 0.35 mL/min) was realized varying the composition of
16
17 183 the mobile phase linearly from 88 % A (ammonium acetate 0.01M solution,
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19 184 acidified to pH 4.5 with acetic acid glacial) and 12 % B (acetonitrile) to 82 % A
20
21 185 and 18 % B at 12 min. The limit of quantification (LOQ) was 155 µg/Kg.
22
23 186 (Gratacós-Cubarsi et al., 2006a).

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26
27 187 Muscle and liver extracts were analysed on a Synergy[™] MAX-RP 4µm (150
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29 188 mm x 3.0 mm i.d.) analytical column (Phenomenex[®], Torrance, CA-US). Elution
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31 189 of SA was realized with binary linear gradient (flow rate 0.75 mL/min) between
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33 190 solvent A (ammonium acetate 0,01M, pH 4.5) and solvent B (acetonitrile). The
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35 191 LOQ was 55 µg/Kg for all matrices (Gratacós-Cubarsi et al., 2006b).

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39 192 The identification of SMZ peak in hair and edible tissues extracts was made
40
41 193 comparing both retention time and spectra (collected between 240 and 400 nm)
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43 194 with those of the SMZ pure standard solutions. SMZ was quantified at a
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45 195 wavelength of 270 nm (16 nm bandwidth) with a reference signal of 450 nm (80
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47 196 nm bandwidth) using an external standard calibration curve.

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50 197 All samples (hair, muscle and liver) were extracted and injected in duplicate.

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55 199 *Statistical analysis*
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3 200 The results of SMZ analysis in pig samples were submitted to a two-way
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5 201 ANOVA and Tukey test, considering the “kind of matrix” (liver, muscle, non-
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7 202 pigmented hair) and animal as independent variables.
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10 203 Data from calf samples were evaluated with two separated three-way ANOVA
11
12 204 and Tukey tests. In the first analysis only hair samples were considered, setting
13
14 205 animal, sampling point and hair colour as independent variables. In the second
15
16 206 analysis the results obtained from samples taken at slaughter were evaluated,
17
18 207 with animal, treatment (WP, NoWP) and kind of matrix (liver, muscle, pigmented
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20 208 hair ad non-pigmented hair) as independent variables.
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24 209 Control sample (C1, C2) and WP1, NoWP1 samples were not considered.
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27 210 Residual concentration of SMZ in the different samples was always the
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29 211 dependent variable; replicates of analysis were taken into account. All the
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31 212 statistical treatment was carried out with the software package Statistica 5.0
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33 213 (StatSoft® Inc. Tulsa, OK-US).
34
35

36 214

37 38 215 **Results**

39 216 *SMZ accumulation in hair*

40
41 217 None of the hair samples obtained from “Control” animals (C1, C2) or taken
42
43 218 before the pharmacological treatment (WP1, NoWP1) contained detectable
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45 219 residues of SMZ. On the contrary, SMZ residues were detected, in a range from
46
47 220 0.4 to 84.7 mg/kg, in the hair samples obtained from treated calves and pigs
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49 221 (Table 1). Figure 2 shows the typical chromatographic profile of contaminated
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51 222 pig and calf hair extracts.
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3 224 The mean SMZ levels in non-pigmented hair of pigs and calves not having the
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5 225 withdrawal period (NoWP2) comprised between 15.9 and 40.5 mg/Kg in pig and
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8 226 between 7.2 and 24.0 mg/Kg in calves (Table 1).
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11
12 228 The SMZ accumulation in hair samples was significantly influenced ($p<0.01$) by
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14
15 229 the inter-individual variance both in pigs and calves. The quantities of SMZ
16
17 230 accumulated in calf hair samples were also significantly influenced ($p<0.01$) by
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19
20 231 both sampling point and hair color. The mean concentration of SMZ detected in
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22 232 pigmented hairs was higher than the corresponding non-pigmented samples,
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24 233 with the exception of the samples taken one week after the beginning of the
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26 234 pharmacological treatment (NoWP2).
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31 236 *SMZ content in hair, muscle and liver*

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33
34 237 The comparison of the SMZ concentration in hair, muscle and liver at slaughter
35
36 238 are shown in Figure 3. The statistical analysis indicates a significant effect
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38 239 ($p<0.05$) of the kind of sample matrix taken at slaughter on the SMZ residual
39
40
41 240 content for both pigs and calves. As showed in figure 3, at slaughter the SMZ
42
43 241 concentration found in hair samples of pigs and calves which did not have a
44
45 242 withdrawal period (NoWP2) was higher than those found in the corresponding
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47
48 243 muscle or liver samples taken from the same animals. At the same time, SMZ
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50 244 residues were found in hair samples of calves having a withdrawal period
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52 245 (WP3), but not in the corresponding edible tissues.
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58 247 **Discussion**
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3 248 The levels of SMZ residues found in calf and pig hair samples are in agreement
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5 249 with those reported by Dunnet & Lees (2004b), who detected SA residues up to
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7
8 250 25 mg/kg in racehorse tails from treated animals. The similar levels of SMZ
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10 251 residues in calf and pig hairs is quite a surprising result, taking into account the
11
12 252 higher quantities of SMZ per kg of live weight that were administered to pigs
13
14
15 253 (about ten fold more than calves), but the different type of SMZ administration
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17 254 (oral in pigs and subcutaneous in cattle) should also be considered as it may
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19 255 have influenced the drug bioavailability (USP DI), the maximum plasmatic levels
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21 256 and finally the drug deposition in hair.
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27 258 The high variability observed for the SMZ accumulation in hair of different
28
29 259 animals, even if statistically significant, should be further investigated with
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31 260 specific studies on a larger population. Anyway, several factors (drug
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33 261 metabolism, hair growth rate, hair composition, feed intake, live weight), which
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35 262 may vary significantly between animals, have been previously described as
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37 263 affecting the drug deposition rate in hair (Henderson, 1993).
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43 265 Pigmented hairs accumulated higher quantities of SMZ than the non-pigmented
44
45 266 ones, in agreement with previous studies which underlined the binding capacity
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47 267 of SMZ (a basic drug) with the melanin pigments to form adducts (Dunnett and
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49 268 Lees, 2004b). Moreover, in non-pigmented samples (WP2, WP3) and in the
50
51 269 new growing samples (NG1, NG2) the concentration of SMZ progressively
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53 270 decreased in the weeks following the treatment, while the corresponding
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55 271 pigmented calf hair gradually accumulated more SMZ residues. This different
56
57 272 time-course in pigmented and non-pigmented samples could be explained
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3 273 considering both the binding capacity of the melanin and the multiple
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5 274 mechanisms which may control the drug deposition in the hair shaft
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8 275 (Henderson, 1993). According to the literature on this matter, if drug
9
10 276 incorporation follows the “passive diffusion” model, the drug concentration in the
11
12 277 blood is the primary factor which controls deposition (Henderson, 1993). As the
13
14 278 drug molecules diffuse from blood to the growing cells of the follicle they are
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16 279 trapped in the hair structure during the keratogenesis. After a variable length of
17
18 280 time from the drug administration the portion of hair containing the drug residue
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20 281 appears over the skin surface and can be sampled and analyzed. A more
21
22 282 complex mechanism has also been suggested - “multiple pool” model - to take
23
24 283 into account that drugs may also be transferred to hair shaft from sweat and
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26 284 sebum or from external environment after the hair has emerged from the skin
27
28 285 (Henderson, 1993). Considering that the average growth rate of calf hair is of 6-
29
30 286 7 mm/month and that the skin thickness is 2.5-3.0 mm (Durant et al., 2002), our
31
32 287 experimental data are in agreement with the hypothesis that the prevalent
33
34 288 mechanism for SMZ accumulation in calf hairs is the formation of bonded forms
35
36 289 with the melanin during the keratogenesis in the follicles under the skin
37
38 290 (“passive diffusion”). Other ways of incorporation from the surface of the skin
39
40 291 can justify the premature detection of SMZ residues in calf hair 7 days after the
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42 292 administration (NoWP2) and are probably the predominant mechanism in non-
43
44 293 pigmented hairs.
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54
55 295 Finally, a very interesting result is that, when SMZ residues were not more
56
57 296 detected in edible tissues of calves which had the withdrawal period (WP3),
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59 297 they were found at mg/Kg levels in both pigmented and non-pigmented hairs of
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3 298 the same animals, indicating a good capacity of hair analysis for the
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5 299 retrospective detection of SMZ administration.
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10 301 **Conclusions**

11
12 302 These results demonstrate that SMZ was accumulated in calf and pig hair in a
13
14 303 stable way at ng/mg level, more than in the corresponding edible tissues.

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16
17 304 Furthermore, SMZ residues have been detected in calf hair since the first seven
18
19 305 days and up to four weeks after the subcutaneous administration, when SMZ
20
21 306 residues were no longer detected in edible tissues of the same animal.
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26
27 308 The high concentration of SMZ residues (mg/kg) accumulated in hair simplifies
28
29 309 the interpretation of analytical results, one of the principal drawbacks of hair
30
31 310 analysis. Even if more studies are required to establish the possible
32
33 311 relationships between administered dose and quantities of SMZ accumulated in
34
35 312 hair, these data show that hair analysis may be a complementary tool in residue
36
37 313 monitoring plans. Hair analysis allows a rapid and cost-effective screening for
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39 314 SMZ administration and could improve the time window in retrospective
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41 315 detection of SMZ administration to food producing animals.
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48 317 Hair analysis can be carried out easily as screening procedure on a large
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50 318 number of animals directly in the farms. Positive hair analysis results, if not
51
52 319 justified by veterinary prescriptions, could indicate how increase or modify the
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54 320 official sampling in a specific farm or group of animals, improving the efficacy of
55
56 321 control and surveillance plans on the edible tissues and discouraging the
57
58 322 improper uses of veterinary drugs.
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324 Finally, future studies will be carried out to verify if other SAs, structurally very
325 similar to SMZ, show also comparable results in terms of residue accumulation
326 in the hair shaft.

327

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60**Figure captions**

430
431 **Figure 1.** Graphic representation of the experimental groups of animals and
432 sampling scheme (WP= having Withdrawal Period; NoWP= Not having
433 Withdrawal Period).

434 The grey zones  correspond to the SMZ administration.

435 Arrows indicate a sampling point.

436

437 **Figure 2.** Chromatographic profiles corresponding to hair samples of animals
438 treated with SMZ: a) pigmented calf hair; b) non-pigmented pig hair.

439

440 **Figure 3.** Mean SMZ residual levels in hair, muscle and liver of treated animals
441 at slaughter.

442 Small letters within the same specie indicate differences at $p < 0.05$ between
443 mean values.

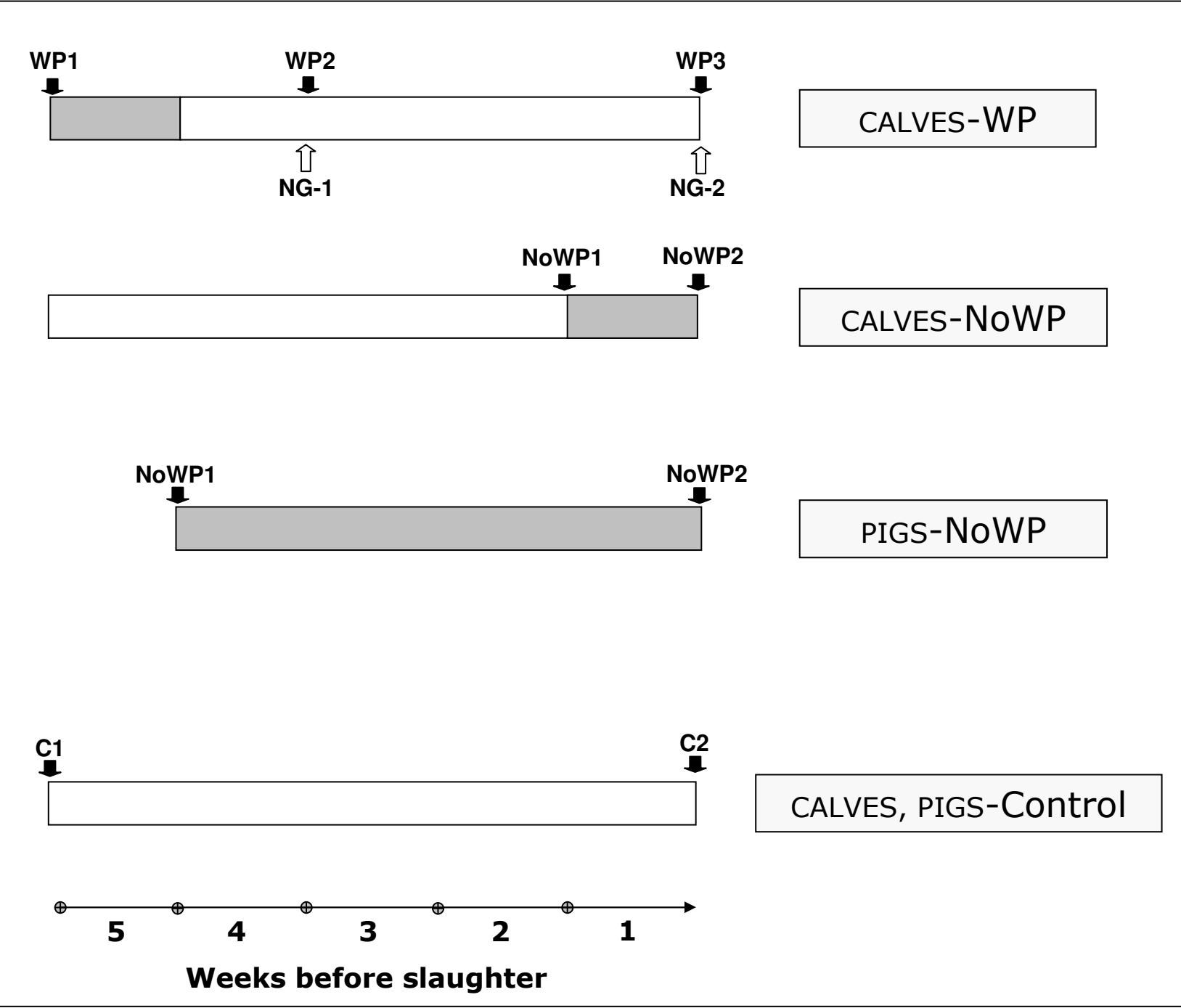
444 Data sets from pigs and calves were submitted to separate statistical tests
445 (ANOVA and Tukey test) as described in material and methods.

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Figure 1



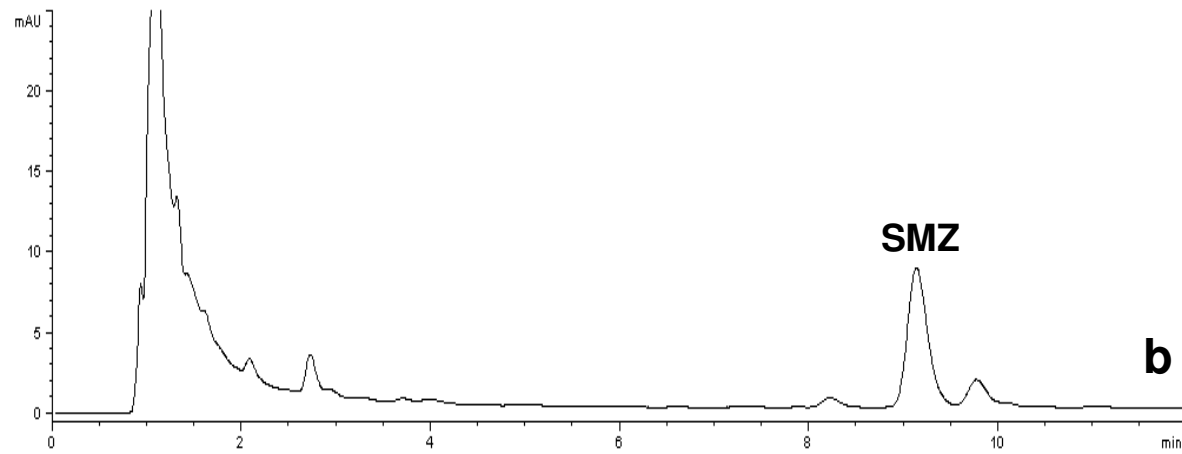
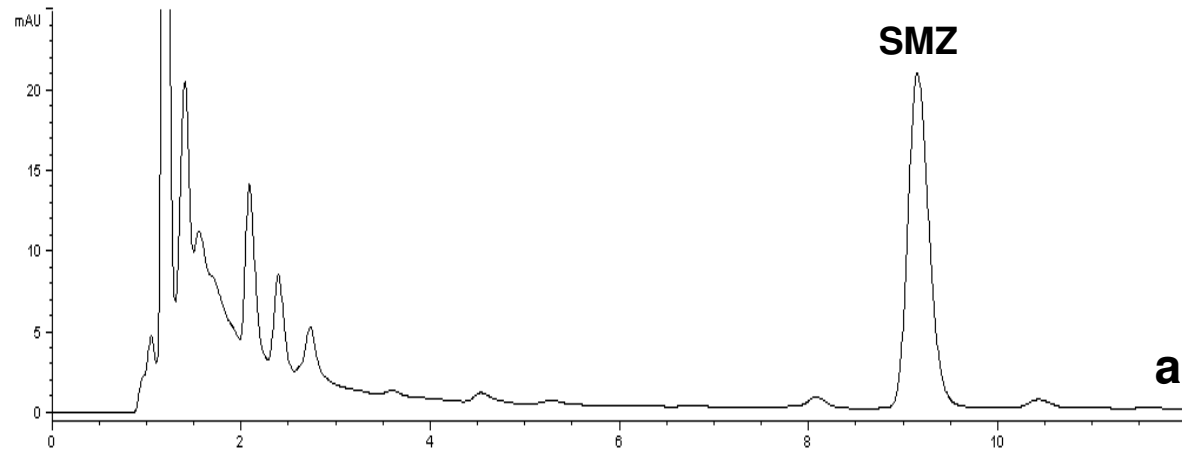
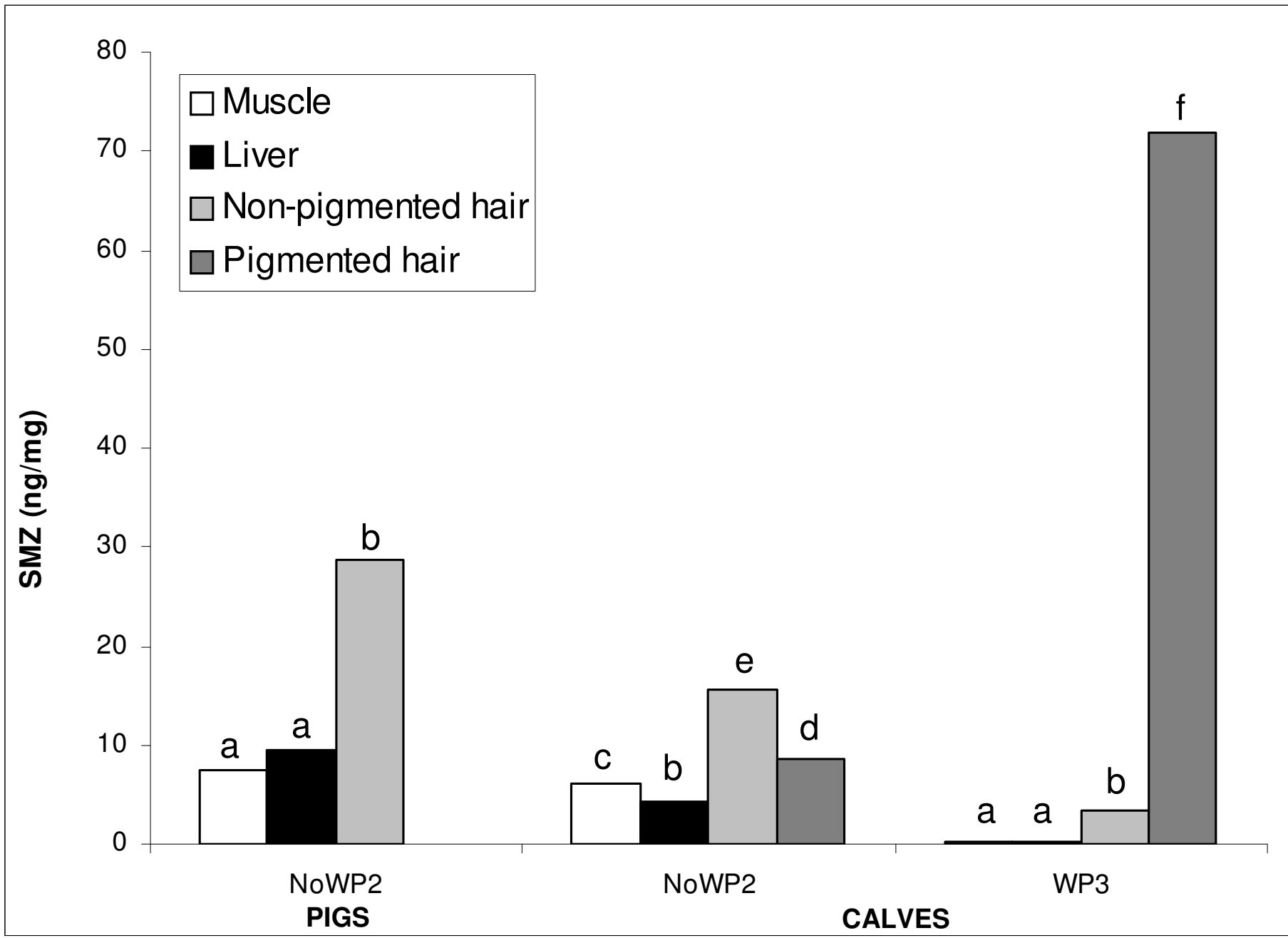


Figure 3



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Table 1. SMZ level (mg/Kg) detected in different hair samples.

Hair sample			NoWP2		WP2		WP3		NG-1		NG-2	
			Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
Non-pigmented	Pigs	Animal 1	40,5	3,7								
		Animal 2	15,9	1,4								
		Animal 3	29,3	2,3								
	Calves	Animal 1	7,2	0,5	7,1	1,6	1,1	0,2	4,2	0,6	1,5	0,3
		Animal 2	24,0	1,5	16,8	0,6	5,6	0,7	1,4	0,6	0,4	0,2
	Pigmented	Calves	Animal 1	5,1	0,7	22,9	4,8	68,5	2,1	37,9	3,6	80,4
Animal 2			12,2	1,5	59,2	1,5	75,4	1,2	84,7	2,2	63,7	2,2