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Detection of sulphamethazine residues in cattle and pig hair by HPLC–DAD

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Abstract

An HPLC method with diode array detection (DAD) is proposed for the detection of sulphamethazine (SMZ) residues in pig and cattle hair. Hair samples were extracted under alkaline conditions (NH_4OH 0.2 M for calf samples and NaOH 0.1 M for piglet samples) and purified with a dual solid-phase extraction (SPE) cartridge system (reverse phase/strong-cation exchange). Recovery of SMZ in fortified samples varied from 70 to 85%, with a limit of quantification of 0.155 ng/mg. Residues of SMZ (7.2–59.2 ng/mg) were detected both in calf and piglet hairs after a therapeutic treatment with SMZ, while no interfering peak was observed in samples from untreated animals.
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1. Introduction

Sulphonamides (SAs) are an important group of antibacterial drugs that have been used in human and veterinary medicine for over 70 years. Various SAs are used in the treatment of, and prevention from, animal diseases, but can also be employed illegally to enhance feed efficiency, animal growth and productivity [1,2].

The misuse of SAs in farming practice can create environmental contamination as well as the development of specific resistance in microbial populations [3–5]. At the same time, the presence of SAs residues in food commodities represents a potential risk to consumer health. Sulphamethazine (SMZ), one of the most used SAs, has been proved to induce tumours in the thyroid gland of rodents [6] and can also produce allergic phenomena to some sensitive consumers [7]. To protect the consumer's health, the European Union established an SAs maximum residue level (MRL) of 100 μg/kg in foodstuffs of animal origin, for all livestock species [8].

The target samples commonly taken for the official controls at the time of slaughter are meat, kidney, fat and liver. The analysis of these samples can show if the withdrawal (28–32 days) was complied with, but are hardly effective at detecting an illicit use of SAs during the first growing phases. In a similar way the analysis of samples taken from live animals (milk, urine, faeces) can only reveal a recent administration of SAs, due to the limited half-life of SAs in biological fluids (less than 17 h for all animal species) [9].

More recently, hair analysis was proposed as a possible technique to increase the time window in retrospective detection of veterinary drug residues.

Hair is a tissue with a lower metabolic activity compared to other conventional biological samples (urine, blood and tissues) and this could preserve the drug residue from degradation once accumulated in the hair shaft. Furthermore hair offers at the same time the advantage of non-invasive collection [10] and can be easily transported and stored. Following this principle, the deposition of anabolic hormones and β-agonists was confirmed in bovine hairs [11–15] as well as the accumulation of different antibiotics in racehorse hair [16,17]. When hair samples are analysed the extraction and purification steps should be specifically studied. Structure and composition of the hair shaft, animal species, pigmentation, and mechanism of drug incorporation can influence the extractability of the veterinary drugs during the analytical process [13,18,19].

Extraction procedures generally include the hair digestion (alkaline, acid and enzymatic) or the direct extraction of the target analyte with organic solvents and/or aqueous buffers.

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Extracts should then be purified, due to the great quantity of impurities contained in these extracts, and concentrated to improve the detectability [13,15–17,20–23].

Various factors must be taken into account and optimised, because of the small quantities of sample to be extracted (few mg_s), the possible degradation of the compounds during the digestion and the complex composition of extracts, in particular if they are obtained by alkaline or enzymatic digestion, can cause serious difficulties in the following LC analysis [16,22].

Since, to the best of our knowledge, the deposition of SAs has only been described in samples of horsehair, the main objective of this study was to demonstrate the accumulation of SMZ in cattle and pig hair after a veterinary administration of this drug. As a consequence, the definition of a suitable protocol for the detection of SMZ residues in cattle and pig hair by LC with diode array detection (DAD) has been developed. Various factors, which can affect the SMZ extraction efficiency, such as sample pre-treatment (pulverisation) and alkaline extraction, were studied using incurred and spiked hair samples. A dual solid-phase extraction (SPE) clean-up, using reverse phase and strong-cation exchange (RP/SCX) mechanisms, was also proposed to increase the limit of detection and to improve the extract purification.

2. Experimental

2.1. Reagents

Sulphamethazine [4-amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-benzenesulphonamide] (minimum 99%), potassium phosphate and ammonium hydroxide ACS reagents were purchased from Sigma–Aldrich (Madrid, Spain). Organic solvents (*n*-hexane, methanol, ethyl acetate and acetonitrile, all gradient grade for liquid chromatography), ammonium acetate, hydrochloric acid, orto-phosphoric acid and glacial acetic acid were obtained from Merck (Darmstadt, Germany). Sodium hydroxide was purchased from Panreac (Barcelona, Spain). Ultra-pure water was obtained using an E-pure system (Barnstead Thermolyne, Dubuque, IA, USA).

Two different veterinary drugs, both containing sulfamethazine sodium salt, were obtained from local stores: Copysulf (injectable solution 330 mg/ml, from FEDRA S.A., Girona, Spain) and Torul S (soluble powder 550 mg/g, from Lamons, Tarragona, Spain).

A sulphamethazine standard stock solution (1 g/l) was prepared by dissolving 100 mg of pure standard in 100 ml of methanol. Spiking solutions for recovery studies (100 and 10 mg/l) were prepared diluting the standard stock solution in methanol. Both standard stock and spiking solutions were stored at −37 °C. Working standard solutions in the range from 0.01 to 2 μg/ml were prepared weekly by dilution of the standard stock solution in ammonium acetate 0.01 M pH 4.5: acetonitrile (85:15, v/v) and stored at 4 °C.

SPE RP polymeric cartridges (HLB-Oasis, 6 cc/200 mg) and mixed mode cartridges (MCX-Oasis, 6 cc/150 mg) were obtained from Waters Corp. (Barcelona, Spain). Strong cationic exchange cartridges (SCX II Accubond, 6 cc/500 mg) were obtained from Agilent Technologies S.L. (Madrid, Spain).

2.2. Animal treatment

Four Friesian calves were reared to 8 months under controlled conditions at the IRTA experimental farm (Prat de Llobregat, Spain) without receiving any medication containing SMZ. In the final 5 weeks prior to slaughter they were divided into two groups, and maintained in separate stalls during the last growing phase.

The first group (Control) did not receive any veterinary drug containing SMZ. The second group (Treated) was supplied, complying with a withdrawal period of 28 days, with 150 mg sulphamethazine sodium salt/kg live weight (daily subcutaneous dose of 40 ml Copisulf solution per animal for 5 days). The treated animals received the daily dose in different zones of the thorax to avoid the development of epithelial lesions.

Six Landrace pigs were also reared to 3 months at the same experimental farm without receiving any drugs containing SMZ. In the final 4 weeks prior to slaughter, a group of three animals (Treated) received an SMZ dose of 1.65 g sulphamethazine sodium salt/kg live weight (daily oral dose of 5.5 g of Torul S mixed with feed for 28 days). The other group of three animals did not receive any medication (Control).

2.3. Hair sampling and pre-treatment

Samples of pigmented and non-pigmented cattle hair (2.0–3.0 g approximately each) were collected 1 week after the end of the pharmacological treatment with SMZ.

Samples of colourless pig hair were also collected from treated and control animals the day of slaughter. All hair samples were collected from the upper area of the neck (to limit external contamination), using a hair clipper Moser Arco 1854 (Wahl Clipper Corp., Sterling, IL, USA) with a cut height of 1 mm. Aliquots of 500 mg of each sample were rinsed three times using 30 ml of ultra-pure water and then dried in an oven at 37 °C for 1 h. Samples were then cut finely with scissors (2 mm approximately) and stored in a desiccator, in the dark and at room temperature until the analysis.

2.4. Extraction and purification of hair samples

2.4.1. Extraction studies

To determine the extraction efficiency a pool of pigmented calf hair samples, obtained from SMZ-treated animals, was homogenised and divided into two sub-samples of approximately 1200 mg each. A sub-sample was then processed (vibration frequency 20 s^{-1}, time 7.5 min) in a Ball Mill MM 200 (Retsch GmbH, Haan-Germany) equipped with a 25 ml grinding steel jar and two grinding steel balls (diameter 12.5 mm) to obtain a fine powder. Both sub-samples were then re-equilibrated in a desiccator and stored in amber glass tubes in the dark and at room temperature until analysis.

Cut and pulverised samples were then extracted using different aqueous alkaline solutions (NH_4OH 0.2 M, NaOH 1 M,

NaOH 0.1 M). Aliquots of 50 mg of pulverized or finely cut calf hair were transferred into 10 ml amber glass tubes and extracted with 4 ml of the different alkaline solutions for variable lengths of time (1, 6 and 24 h) in a water bath at 60 °C (each treatment in duplicate).

A similar procedure was carried out with a pool of hair samples obtained from pigs treated with SMZ. A 1 g sub-sample was pulverised in the Ball Mill (vibration frequency 30 s^{-1}, time 10 min). Aliquots (50 mg) of cut and pulverised samples were then extracted using 4 ml of NaOH 0.1 M or NH_4OH 0.2 M as digesting solutions for 30 min 1, 6 and 24 h at 60 °C (each treatment in duplicate).

2.4.2. Purification studies

Crude alkaline extracts were centrifuged at 2700 g for 10 min (Megafuge 1.0 Heraeus Sepatech, Osterode/Harz-Germany) and filtered through a paper filter. Due to the complexity of these extracts two different SPE purification protocols were essayed.

The first protocol was adapted from Dunnet and Lees [16] with some modifications. Alkaline extracts were added with HCl 1 M and buffered with potassium phosphate solution 0.01 M (4 ml approximately) until pH 6.0. The final value of pH was checked with a micropH 2001 (Crison, Barcelone-Spain) and then the buffered sample re-centrifuged to eliminate the cloudiness. The buffered clear extracts were then loaded onto an Oasis-MCX mixed mode cartridge, previously activated with 4 ml of methanol, 1 ml of ultra-pure water and 4 ml of buffer potassium phosphate monobasic 0.01 M (pH 6.0). Columns were then sequentially washed with 2 ml of buffer potassium phosphate monobasic 0.01 M pH 6.0, 1 ml H_3PO_4 1.8 mM, 2 ml of a 30% methanol aqueous solution and 2 ml *n*-hexane: ethylacetate (80:20, v/v). SMZ was eluted with 4 ml methanol.

The second purification protocol employed a dual SPE cartridge system. Centrifuged alkaline extracts were directly loaded onto an Oasis-HLB cartridge, previously activated with 4 ml of methanol and 4 ml of ultra-pure water. The cartridge was washed with 2 ml of 5% aqueous methanol and then dried under vacuum (2 min). SMZ was eluted with 4 ml acetic acid: acetonitrile (5:95, v/v) and loaded directly onto an Accubond Strong Cation Exchange (SCX) cartridge previously activated with 5 ml acetic acid: acetonitrile (5:95, v/v). The SCX cartridge was washed with 5 ml methanol:acetonitrile (1:1, v/v), then SMZ was eluted with 5 ml of ammonium hydroxide: methanol (5:95, v/v) solution.

Eluates containing SMZ were evaporated under nitrogen at 25 °C, reconstituted with 400 μl of ammonium acetate 0.01 M pH 4.5: acetonitrile (88:12, v/v) and filtered through a 0.45 μm nylon filter.

Recovery studies were carried out adding variable volumes (40, 10 and 2.5 μl) of spiking standard solution (10 μg/l SMZ in methanol) to "Control" hair samples (50 mg), to reach a final SMZ concentration of 8, 2 or 0.5 ng/mg hair. After fortification the samples were equilibrated for 10 min at ambient temperature and then analysed following the described protocols.

Repeatability was evaluated running the complete analysis of contaminated samples three times on the same day.

2.5. HPLC analysis

SMZ analysis was carried out on a 1100 liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a quaternary pump and a diode array detector. Chromatographic separation was performed on a Luna RP-C_{18} (150 mm × 2.1 mm i.d.) column (Phenomenex, Torrance - California, USA) at a flow rate of 0.35 ml/min. A binary gradient elution was realized varying the composition of the mobile phase linearly from 88% A (ammonium acetate 0.01 M solution, acidified to pH 4.5 with acetic acid glacial) and 12% B (acetonitrile) to 82% A and 18% B at 12 min. All the sample extracts were injected in triplicate.

The identification of SMZ peak in hair extracts was made comparing both retention time and spectra (collected between 240 and 400 nm) with those of the SMZ pure standard solutions. Peak purity was also evaluated with the Chemstation software utility. SMZ was quantified at a wavelength of 270 nm (16 nm bandwidth) with a reference signal of 450 nm (80 nm bandwidth). An external standard calibration curve was created by injecting per triplicate known amounts of SMZ (0.1, 1, 2, 5, 10, 20 and 40 ng).

3. Results and discussion

3.1. Extraction studies

Optimization of the extraction conditions was carried out considering different alkaline solutions, at a temperature of 60 °C and a maximum time of contact of 24 h. Higher temperatures (80 °C) and/or longer extractions (48 h) were essayed in preliminary trails but discarded because they did not improve the extraction significantly. Similar results were observed by Dunnet and Lees [16] in horsehair, where the majority of sulphonamides were extracted within the first 24 h. In a similar way, acidic digestion (HCl 1 M) or the use of methanol as the extracting medium were finally not considered because poor extraction efficiencies were observed.

Fig. 1a and b show the extraction kinetic of SMZ of a pigmented incurred calf hair sample. The characteristics of alkaline solution clearly affect the extraction of SMZ from hair samples. The higher extraction efficiency was obtained with NH_4OH 0.2 M and 24 h of contact at 60 °C. These digestion conditions produced a clean extract, even operating with pigmented hair samples.

When sodium hydroxide solutions were used, a lower extraction of SMZ from hair was observed, especially with the 1 M solution, probably due to a partial degradation of the SMZ during the digestion. Moreover, the use of NaOH solutions complicated the clean-up and the chromatographic separation, due to the migration of many interfering compounds in the extracts, as previously observed by other authors [16,22]. Under our conditions the pulverisation did not produce any significant improvement, so the final conditions for the extraction of calf hair combined the digestion for 24 h with NH_4OH 0.2 M and finely cut hair.

The pig hair samples required the development of a specific extraction protocol. Preliminary trials showed that the hard structure of these samples needed a pulverisation step to improve

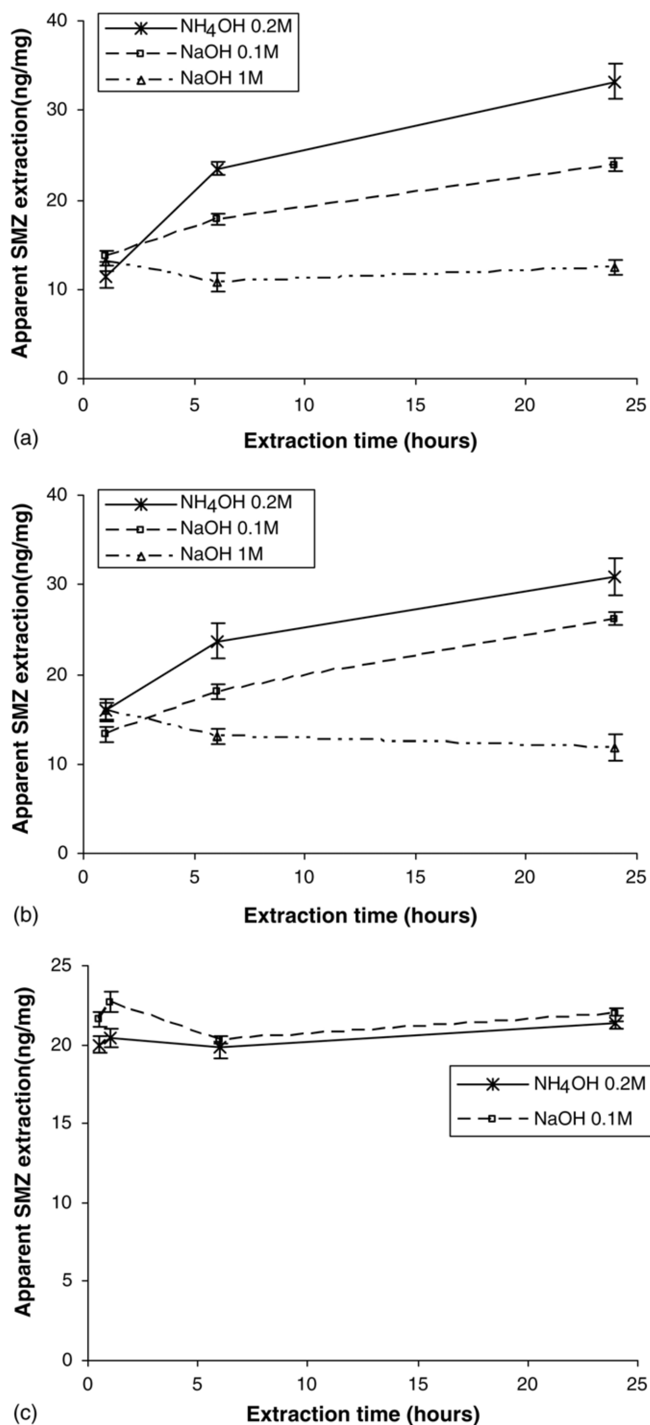

Fig. 1. Extraction time-course profile of SMZ from finely cut calf hair (a), pulverised calf hair (b) and pulverised pig hair (c) with different alkaline solutions.

the extraction efficiency and the homogeneity of the sample. On the other hand the use of NaOH 1 M reduced the extraction efficiency drastically.

For these reasons the overall kinetic of extraction was evaluated considering merely pulverised samples and alkaline solutions containing NaOH 0.1 M or NH_4OH 0.2 M (Fig. 1c). The higher extraction was observed using the NaOH 0.1 M solution and an extraction time of 1 h. Longer times of extraction did not improve the extraction significantly. At the same time the crude NaOH pig extracts always appeared clearer and less coloured than the calf ones, and could be purified and analysed easily.

3.2. Purification

The first protocol developed for the clean-up of the alkaline extracts was adapted from that proposed by Dunnet and Lees [16] based on mixed-mode SPE cartridges (C_{18}/SCX). An additional washing with 30% methanol aqueous solution was added to the original procedure to improve the clean-up due to the presence of many interfering compounds in alkaline calf hair extracts. Under these conditions a satisfactory SMZ recovery (75–80%) and a good clean-up was obtained. On the other hand we observed the pH of the extracts, if not corrected precisely, could cause poor recovery and reproducibility. So, a pH-meter was used to check the addition of HCl 1M and buffer phosphate to the extract, although this procedure is time-consuming and could introduce a source of cross-contamination.

To avoid these problems a second protocol was developed operating a dual SPE clean-up (RP/SCX). The alkaline extract (pH approximately 12) was loaded onto a SPE Oasis-HLB directly. Under these conditions the SMZ molecule was strongly retained in the stationary phase. Polar impurities were eliminated by washing with an aqueous 5% methanol solution. SMZ was then eluted under acidic conditions (pH 4.0) and loaded directly onto the SCX cartridge. At pH 4.0 the SMZ molecule was positively charged and neutral/basic impurities were eliminated washing the cartridge with methanol/acetonitrile. The final elution with NH_4OH:methanol allowed the quantitative recovery of SMZ and the evaporation of the liquid phase to re-dissolve the sample in a small volume of LC mobile phase. Using this protocol a very satisfactory purification of hair extracts was obtained and furthermore the extract manipulation and the time of analysis were reduced.

Table 1 summarises the results of the recovery studies on spiked pig and calf hair samples for the whole analysis with the dual SPE protocol. Repeatability results were always better than 6.2% while average recovery was higher than 80% in pig samples and within 70–78% in calf samples. Chromatographic response was linear ($r^2 = 0.9998$) in the range between 0.1 and 40 ng of SMZ injected.

Table 1
Recovery (mean value ±S.D.) and repeatability (mean value and R.S.D.) data obtained for the whole analysis with fortified and incurred pig and calf hair samples

Sample type	Accuracy			Repeatability	
	Spiking level (ng/mg)	Recovery		SMZ (ng/mg)	R.S.D.[a]
		(%)	(±) S.D.[a]		
Colourless pig hair	8.0	81.2	2.1	37.5	6.2
	0.5	85.5	0.7		
Pigmented calf hair	2.0	78.2	0.4	29.2	5.5
	0.5	72.3	1.9		
Colourless calf hair	2.0	69.7	1.8	11.5	2.4
	0.5	75.6	5.4		

[a] ($n = 3$).

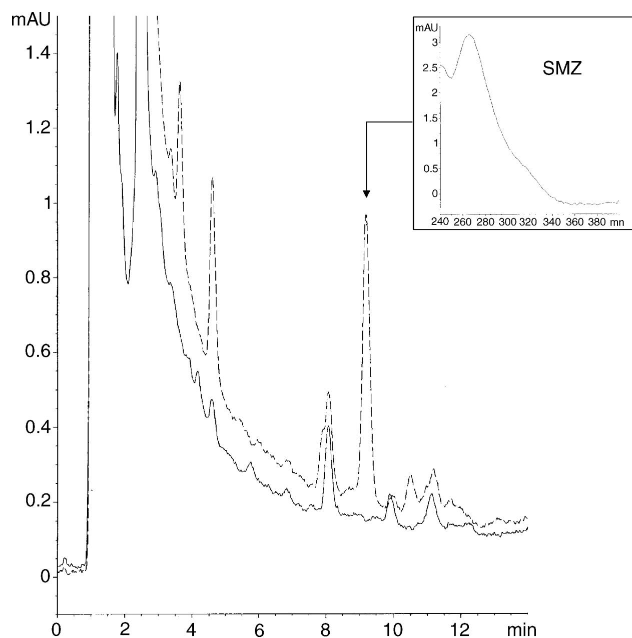

Fig. 2. HPLC chromatograms of 50 mg “Control” calf hair sample (—) and 50 mg “Control” calf hair sample spiked with 2 ng/mg SMZ (- - -). (5 μl injected for each extract).

The Limit of Detection (LOD_{SMZ} s/n = 3), calculated taking into account the chromatographic LOD, the recovery rate and the sample dilution was 0.046 ng/mg injecting 20 μl of sample extract. At the Limit of Quantitation (LOQ_{SMZ} s/n = 10; 0.155 ng/mg) a satisfactory evaluation of the peak spectra was still possible.

Fig. 2 shows the chromatographic separation obtained from a calf “Control” hair sample and from the same hair sample fortified with 2 ng/mg of SMZ. Under our chromatographic conditions the SMZ peak showed an average t_R of 9.3 min (R.S.D. 2%, $n = 12$). The SMZ peak showed a good chromatographic shape (peak symmetry always better than 0.78) and no interfering peaks were detected in the extracts obtained from the “Control” samples.

The typical chromatographic separations corresponding to hair samples of “Treated” calves are shown in Fig. 3. Accumulation of “mother” drug was observed in both pigmented and colourless hair samples collected 1 week after the end of treatment with SMZ. The quantities varied between 22.9 and 59.2 ng/mg in pigmented samples, and between 7.2 and 12.3 ng/mg in colourless samples.

The deposition of SMZ was also assessed in the case of the hair obtained from “Treated” pigs. Fig. 4 resumes the typical chromatograms obtained with “Control”, spiked and incurred samples. SMZ was detected in all the hair samples collected from “Treated” animals (15.9–40.5 ng/mg), while no interfering peak with t_R near to SMZ was observed in “Control” samples.

It is well known that sulphonamides, once administered, are rapidly transformed to oxidised, acetylated and hydroxylated derivatives. The N4-acetyl derivatives are the principal sulphonamide metabolites observed in pigs [7,9].

Under our conditions, a peak with t_R of 9.8 min, absent in “Control” and fortified samples and with a spectra similar to

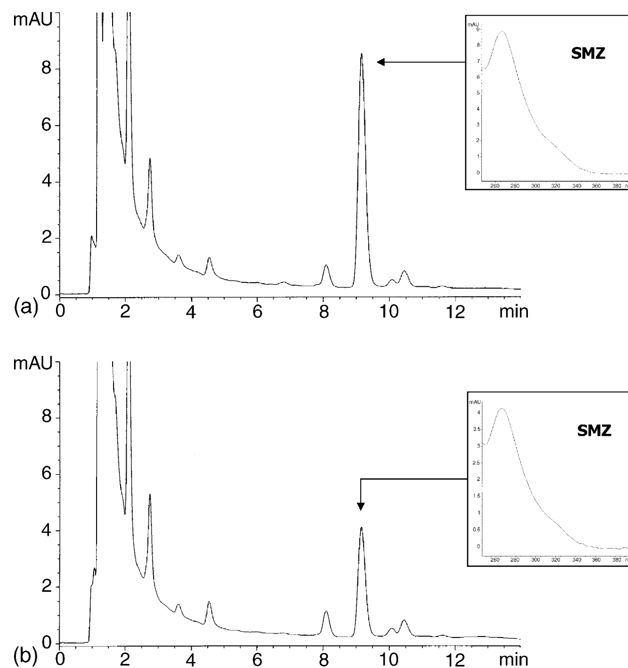

Fig. 3. HPLC chromatograms of incurred calf hair: (a) 50 mg pigmented hair sample (SMZ = 22.9 ng/mg) and (b) 50 mg colourless hair sample (SMZ = 12.3 ng/mg) (5 μl injected for each extract).

SMZ’s, was also observed in all “Treated” samples. Even if more studies (LC–MS) are required to confirm the nature of this compound, this peak could correspond to some SMZ metabolite accumulated in hair during the bio-metabolic processes, as observed in horses [16].

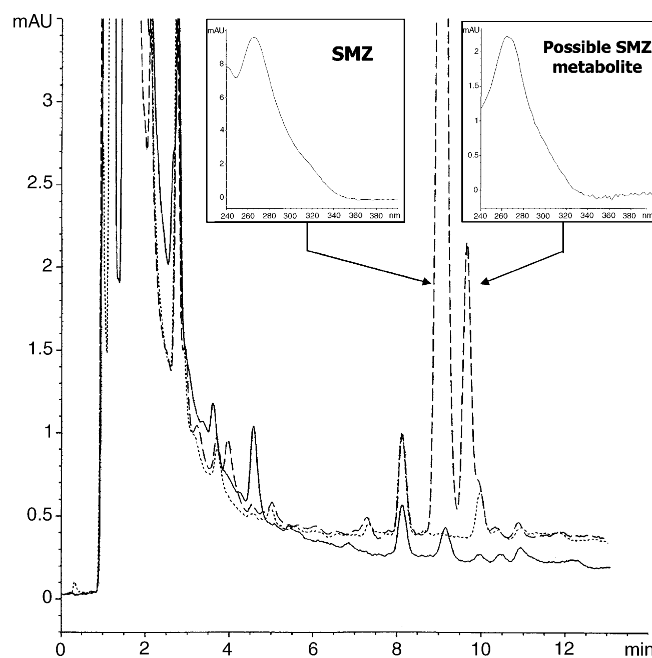

Fig. 4. HPLC chromatograms of 50 mg incurred pig hair sample* (- - -), 50 mg “Control” pig hair sample (· · ·) and 50 mg “Control” pig hair sample spiked with 0.5 ng/mg SMZ (—). Five microliters injected for each extract. *[SMZ = 30.2 ng/mg].

4. Conclusions

An HPLC–DAD analytical method, including a novel dual SPE purification, was developed for the detection of SMZ residues in pig and cattle hair. The suitability of this method was verified on spiked and incurred hair samples. Residues of SMZ were detected, at ng/mg level, in hair samples of both calves and pigs supplied with therapeutic doses of SMZ. Accumulation in the hair structure of a possible SMZ metabolite was also observed in hair samples obtained from pigs treated with SMZ. The presence of this metabolite, if confirmed, could be a suitable way to corroborate the improper use of SMZ, to discard external contamination or to determine the method of drug administration (oral, parenteral) [17,24].

These results indicate that remarkable quantities of SMZ can be accumulated in the structure of pig and calf hair after its administration. More studies are currently being carried out to determine if hair analysis could be effective in the retrospective detection of the SMZ administration when SMZ residues are already undetectable in conventional samples as observed in horses [25].

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