



A rapid HPLC method for the determination of sulphonamides and trimethoprim in feed premixes

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ABSTRACT. A simple HPLC method for the simultaneous determination of sulphadimidine, sulphadiazine, sulphamethoxazole and trimethoprim antibiotics in medicated feeds has been developed, validated, and applied to commercial feed premixes. Ultrasound-assisted extraction was used for the recovery of the studied antibiotics without the need for a further clean-up step. The sulphonamides and trimethoprim were separated on a Symmetry C18 (5 µm, 4.6 × 250 mm) column using 10-min isocratic elution and UV detection at 254 nm. The results showed that the method is robust with adequate recovery (minimum 95.19%), satisfactory repeatability (1.39%–2.69%) and intermediate precision (1.36%–4.16%), as shown by the validation assessment herein. The advantages of the proposed method are the speed, the ease of the clean-up protocol, and the low cost of the needed instrumentation, rendering it highly applicable to the feed industry.

Introduction

The use of medicated feeds (i.e. feedstuffs that include prescribed medicinal products) is the main administration method in intensive farming and the most effective way for a farmer to give medicine to his livestock (Turnidge, 2004). Mixes of sulphonamides (SAs) and trimethoprim (TMP) constitute the second most commonly used antimicrobials in authorized premixes in the European Union because of their broad spectrum antibacterial activity for the treatment of respiratory and/or gastrointestinal tract infections in sheep, cattle and pig production (EU Commission, 2010). Due to the awareness of the development and the spread of antibiotic resistance,

however, alternatives to medicated feeds have been proposed (Link et al., 2005; Grashorn, 2010), while the legal framework of the EU relating to the use of antimicrobials in feeds is extremely rigorous (EU Commission, 2010). Therefore, the use of validated methods for the simultaneous determination of sulphonamides and trimethoprim in feed premixes is of high practical issue for different stakeholders in feed production, including the feed industry, inspection authorities, or even authorized farm mills.

Although several analytical methods are reported in the literature for determination of SAs, alone or in combination with TMP in different biological fluids (Teshima et al., 2004), foods (de Paula et al., 2008), or environmental samples (Cahill et al., 2004), only

a limited number of analytical protocols for feeds has been published (Samanidou and Evaggelopoulos, 2008). The methods investigating feed premixes are mainly based on enzyme-linked immune-absorbent assays, ELISAs (Jimenez et al., 2009), or high performance liquid chromatography methods, combined or not, with tandem mass spectrometry (Croubels et al., 2002; Boscher et al., 2010; Lopes et al., 2012). Nevertheless, the methods suggested to date for the simultaneous determination of SAS and TMP require either a long and tedious analysis protocol (DeAngelis et al., 1990; Renew and Huang, 2004), or more explicit instrumentation, like an FT-IR spectrometer (Silva et al., 2009), capillary electrophoresis system equipped with photodiode array detector (Farooq et al., 2009), or LC-MS/MS set-up (Boscher et al., 2010; Lopes et al., 2012).

The scope of the present study was to develop and validate a straightforward, low-cost and simple HPLC method for the simultaneous quantification of sulphadimidine (SDM), sulphadiazine (SDZ), sulphamethoxazole (SMZ) and TMP for routine analysis of feed premixes. Furthermore, time-consuming and laborious pre-treatment procedures were to be avoided in order to propose a widely applicable routine analysis method.

Material and methods

Chemicals. Sulphadiazine (4-amino-N-pyrimidin-2-ylbenzenesulphonamide), sulphamethoxazole (4-amino-N-(5-methyl-3-isoxazolyl) benzenesulphonamide), sulphadimidine (4-amino-N-(4,6-dimethylpyrimidin-2-yl) benzenesulphonamide), and trimethoprim (5-(3,4,5-trimethoxybenzyl) (pyrimidine-2,4-diamine) were obtained from Sigma Aldrich (Figure 1). Triethylamine and glacial acetic acid were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile and water were HPLC grade and provided by Scharlau (Barcelona, Spain). Different commercial feed premixes were kindly offered by Vita Trace Nutrition Ltd.

Sample extraction. The extraction of the premixes was done in triplicate for each sample as follows: 200 mg of premix sample was mixed with 50 ml methanol in an ultrasonic bath for 20 min at room temperature. Then, the extracts were filtered through a 0.20 μm PTFE filter (Waters, Milford, MA) before injection into the HPLC.

Instrumentation. The HPLC system consisted of a binary DIONEX P680 pump equipped with a Dionex DG 1310 Degasser. The samples were injected using a Dionex ASI-100 autosampler.

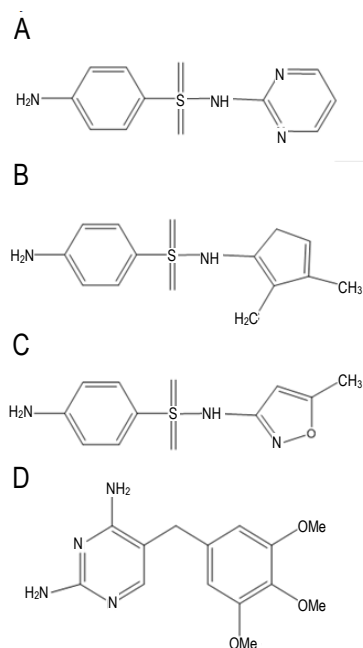


Figure 1. Chemical structures of sulphadimidine (A), sulphadiazine (B), sulphamethoxazole (C) and trimethoprim (D)

Antibiotic monitoring was accomplished at 254 nm using a diode array (DAD) system Dionex UVD 340U.

Chromatographic conditions. The chromatographic separation was carried out on a Symmetry C18 (5 μm , 4.6 \times 250 mm) column. Two litres of the mobile phase were prepared by mixing water:acetonitrile:triethylamine (1400:400:2, v/v/v). The pH of the elution solvent was adjusted to 5.9 using glacial acetic acid and the solution was diluted to 2 l with HPLC-grade water as described for the determination of SMZ and TMP in a previous study for pharmaceutical tablets (Silva et al., 2009). A flow rate of 1.7 ml \cdot min⁻¹ was used for the chromatographic separation.

The accuracy of the method is presented in terms of trueness and recovery. The trueness was determined by preparing and measuring six different samples from each of the raw material samples (SDM, SDZ, SMZ and TMP). The results were used to calculate the SD, relative standard deviation (RSD) and trueness. Recovery was determined by using real samples containing known amounts of the analytes. Five different samples were prepared containing 80%, 90%, 100%, 110% and 120% of each analyte, respectively. Three injections were made for each different concentration on the same day. Recovery was calculated from these results.

The precision of the method was determined in terms of repeatability and intermediate precision. A sample containing 100% of each analyte was used

for the determination of these terms. Repeatability was determined by preparing and measuring six different samples from the real sample within short time intervals. Two injections were made for each of six samples. From results the SD, RSD and the repeatability limit were calculated. The intermediate precision was determined by preparing and measuring a total of twelve different samples from the real samples by two different analysts (6 samples each) on different days. Two injections were made for each of the 12 samples. From these results, SD, RSD and intermediate precision were calculated.

Linearity was determined by measuring five different samples containing 80%, 90%, 100%, 110% and 120% of each analyte. From the results, correlation coefficient and R-square values were calculated.

Results and discussion

The extraction of SAs and TMP from feed premixes or other animal feedstuffs is a crucial step of the overall analytical process that leads to the quantification of targeted antibiotics. Recently, Sun et al. (2007) suggested that polar solvents are more suitable for the extraction of sulphonamides from natural animal casings than organic solvents such as dichloromethane, acetone, acetonitrile and ethyl acetate. Methanol has also been used for determination of

TMP in wastewaters (McClure and Wong, 2007). In the present study, the ability of various polar solvents such as water, methanol, ethanol and hydroalcoholic mixtures such as methanol:water (50:50) and ethanol:water (50:50) to extract the studied antibiotics was tested. Methanol had the highest recovery of antibiotics compared with the other studied solvents. The solubility of SDZ is low in ethanol, while the mixture of ethanol:water (50:50) gives high extractability of antibiotics without adequate selectivity.

The extraction time was also optimized during validation of the proposed method. It was observed that the recovery of the antibiotics was increased by extending the extraction time to 20 min. Additional extraction time, e.g., up to 30 or 60 min, did not further improve the recovery rate of the substances. The extraction of SAS by mixing feed premix sample with methanol using an ultrasonic bath for 10 min was around 90% of the spiking substance, but the repeatability of the extraction was unacceptable. Therefore, ultrasound-assisted extraction with methanol for 20 min was proposed since optimum recovery and satisfactory repeatability had been obtained. Furthermore, the proposed extraction protocol was a rapid, simple, and low-cost method without necessitating a solid phase extraction step as the previously reported methods (Boscher et al., 2010; Kumar and Companyo, 2011; Liu et al., 2011).

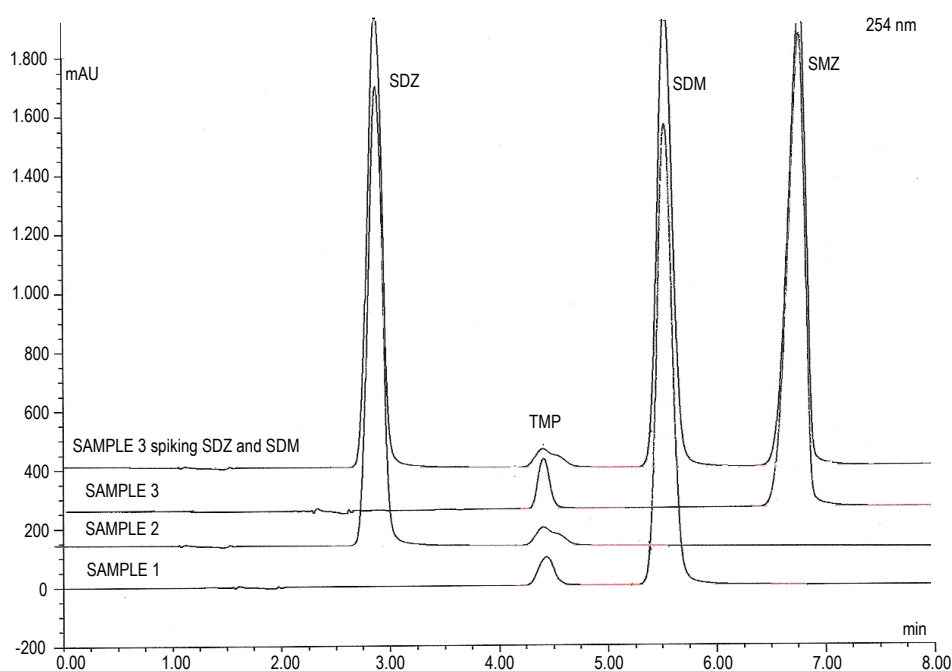


Figure 2. Chromatographic separation of commercial premixes. The chromatogram is recorded at 254 nm and the peaks under study are denoted

The next step was to establish the chromatographic separation of studied antibiotics. Figure 2 shows that the proposed method allows successful separation of different antibiotics in a single run. The duration of chromatographic separation was less than eight min. The limits of detection and quantification were $3 \mu\text{g} \cdot \text{ml}^{-1}$ and $10 \mu\text{g} \cdot \text{ml}^{-1}$ for each antibiotic, respectively. The linear correlation between peak area and concentration of four compounds was determined. Data for five solutions of different concentrations in the range between 0.72 to $1.20 \text{ mg} \cdot \text{ml}^{-1}$ for SDM, 0.80 to $1.19 \text{ mg} \cdot \text{ml}^{-1}$ for SDZ, 0.59 to $0.84 \text{ mg} \cdot \text{ml}^{-1}$ for SMZ, and 0.16 to $0.24 \text{ mg} \cdot \text{ml}^{-1}$ for TMP were collected. The slope, intercept, and correlation coefficient were calculated and the results are listed in Table 1.

Table 1. Linearity of method for the determination of sulphonamides and trimethoprim premixes

| Characteristics | Sulphadiazine | Sulphamethoxazole | Sulphadimidine | Trimethoprim |
|-------------------------|---------------|-------------------|----------------|--------------|
| Slope | 5.78 | 5.19 | 5.72 | 1.84 |
| Intercept | 0.0256 | 0.0014 | 1.09 | 0.0006 |
| SD of intercept | 0.0233 | 0.0012 | 1.17 | 0.0007 |
| Correlation coefficient | 0.9999 | 0.9999 | 0.9998 | 0.9999 |

Table 2. Accuracy and precision of method for the determination of sulphonamides and trimethoprim premixes

| Antibiotic | Accuracy | | Precision | |
|-------------------|------------|--------------|---------------|------------------------|
| | trueness | recovery | repeatability | intermediate precision |
| Sulphadiazine | 99.77±0.89 | 95.19–100.59 | 2.59% | 4.16% |
| Sulphamethoxazole | 99.70±1.22 | 95.74–99.26 | 2.69% | 2.31% |
| Sulphadimidine | 99.08±2.63 | 98.28–99.92 | 1.35% | 1.36% |
| Trimethoprim | 99.72±1.22 | 95.44–98.68 | 1.39% | 1.60% |

The accuracy of the present method was tested by determining the recovery of the antibiotics under study. Five different premixes were prepared containing 80%, 90%, 100%, 110% and 120% of each analyte and their recovery was calculated (Table 2). The recovery ranged from 95.19% to 100.59% for sulphonamides and from 95.44% to 98.68% for trimethoprim. This recovery was comparable with those in previous studies in which an additional solid phase extraction step was used (Boscher et al., 2010; Liu et al., 2011). These findings confirmed that the proposed method is accurate with satisfactory repeatability (relative standard deviation of six replicates). The RSD of sulphadimidine and TMP was 1.35% and the corresponding value for sulphadiazine and sulphamethoxazole was 2-fold lower

(2.59%–2.69%). Boscher et al. (2010) achieved repeatability that ranged between 86.0% and 91.0% for trimethoprim and 86.9% to 91.2% for sulphadiazine, respectively.

Conclusions

In summary, the developed RP-HPLC method was found to be suitable for the separation and quantitative determination of sulphonamides and trimethoprim in medicated premixes or animal feeds. The use of ultrasound-assisted extraction in combination with methanol gave efficient recovery of antibiotics. The proposed assay is straightforward, simple, and applicable to routine analysis, while its accuracy and precision are particularly satisfactory and comparable with more complicated protocols.

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