INTRODUCTION

Fenbendazole, a broad spectrum benzimidazole anthelmintic, has been widely used against all classes of helminths that commonly infect animals. Following oral administration to cattle and sheep, fenbendazole is slowly absorbed from the gut and rapidly transformed to a range of metabolites, with fenbendazole sulphonyl and fenbendazole sulphone being the major ones [1]. Therefore, its administration in these animals may result in the presence of remarkable concentrations of fenbendazole residues in foodstuffs of animal origin, of which milk and dairy products are most important because of their daily consumption.

The presence of fenbendazole residues in dairy products is potentially harmful for the consumers, although fenbendazole is not harmful per se. One of its metabolites, oxendazole, has been proven responsible for teratogenic and embryotoxic effects in laboratory animals when administered during the primary stages of pregnancy [2]. To protect consumers' health from the presence of harmful concentrations of fenbendazole residues in marketed milk, a maximum residue level (MRL) of 10 μg/kg for the marker residue of fenbendazole has been established by the European Union [3].

At present, no analytical methodology is available for the extraction, separation, and quantification of fenbendazole and its sulphone, sulphoxide and hydroxylated metabolites in fermented dairy products. The recently published homologous LC/MS/MS method [4] for the analysis of veterinary drug residues in cheese does not cover the EU prerequisite to consider fenbendazole sulphone and p-hydroxyfenbendazole in the determination. The objective of this study was to develop and validate, according to Commission Decision 2002/657/EC [5], a simple, rapid, sensitive and cost-effective LC method for the accurate and precise determination of the marker residue of fenbendazole in fermented dairy products. The method is aimed to be applied to quantitate fenbendazole and its metabolites in Feta cheese and yoghure made from spiced and incurred ovine milk.

MATERIALS AND METHODS

Sample extraction and cleanup

A 1.5-g sample (yoghurt or grated cheese) was transferred into a 15-mL graduated centrifuge tube to which the extraction solution (mixture of acetonitrile:1H phosphoric acid, 9:1) was added to a final volume of 7 mL. The content was homogenised (20 s) using an Ultra-Turrax and centrifuged for 1 min at 4000g. A 3-mL aliquot of the clear supernatant was transferred into another 15-mL tube and 2 mL of 0.1 M phosphate buffer, pH 11, were also added. After vortex mixing for 10 s, the extract was adjusted to 5 mL of hexane under high speed vortex mixing for 30 s, and centrifuged as described above. The top hexane layer was discarded and following the addition of 5 mL ethyl acetate, vortex mixing for 1 min and centrifugation, the top organic layer separated was transferred into another 15-mL tube to be further purified by vortex mixing (1 min) and centrifugation after the addition of 3 mL of 0.1 M phosphate buffer, pH 11. The supernatant ethyl acetate layer was transferred into another tube and evaporated to dryness under nitrogen. The remaining residue was dissolved in 300 μL of mobile phase and a 50-μL aliquot was submitted to LC analysis.

LC procedure

Separation of fenbendazole and its sulphonyl, sulphoxide, and p-hydroxy metabolites was carried out isocratically using an acetonitrile–0.01 M phosphoric acid (33:67, v/v) mobile phase containing 4.0 mM octanesulphonate sodium salt and 5 mM tetrabutylammonium hydrogen sulphate. Following its preparation, the mobile phase was filtered by passing through a 0.2 μm Nylon-66 filter (Anachem, Luton, UK). The mobile phase was delivered in the system at a rate of 1 mL/min. The Nucleosil 100-5 C18 analytical and guard columns were equilibrated with the mobile phase each time before use and kept thermostatted at 55 °C during runs. Detection was performed at 290 nm using a diode-array detector.

Under these conditions, oxendazole eluted at 5.4 min, p-hydroxyfenbendazole at 7.0 min, fenbendazole sulphone at 8.5 min and fenbendazole at 17.9 min (Figure 1).

Method validation

Validation was performed according to the Commission of the European Communities guidelines (European Commission, 2002) using blank cheese and yoghurt samples. The specificity, linearity, sensitivity, accuracy, precision, applicability, and stability of the analytes were the criteria used to evaluate the developed method.

RESULTS

Method validation

Chromatograms obtained from blank yoghurt and cheese extracts, and from reagent blanks showed that the peaks attributable to test analytes were resolved sufficiently from other peaks (baseline resolution) allowing reliable quantification (Figure 1):

The efficiency of the ion-pair liquid chromatographic system along with the cleanliness of the extracts enabled detection and quantification limits in the range of 3.0 to 8.7 and 9.9 to 20.9 μg/kg, respectively, for all analytes in both yoghurt and cheese samples.

The method exhibited excellent analytical characteristics in terms of precision and accuracy. Overall recoveries of all analytes ranged between 80.5 and 88.8% for yoghurt, and 79.8 and 87.6% for cheese, whereas overall precision (RSD%) for all analytes was lower than 11% for both products.

Storage stability

Oxendazole and fenbendazole sulphone were stable in the mobile phase for at least 36 weeks at all three temperatures (25°C, 4°C, −20°C) examined. p-Hydroxyfenbendazole and fenbendazole were quite stable for 24 weeks at all three temperatures, but a reduction of about 9% and 6% was noted for both analytes after 36 weeks of storage at 25°C, in the light and in the dark, respectively. However, standard solutions of these two analytes in mobile phase were stable for at least 36 weeks when stored at −4 °C and −30 °C. Moreover, the test compounds were stable during storage of fortified and incurred yoghurt and cheese samples at −80°C for at least 12 months, whereas, no significant change in the concentration of all four analytes during storage of final yoghurt and cheese extracts at 25°C for 7 days, was observed.

Applicability

In order to validate the method with real samples, a trial was carried out to quantify fenbendazole residues in Feta cheese that was prepared from ovine milk spiked with oxendazole, p-hydroxyfenbendazole, fenbendazole sulphone and fenbendazole. The analysis results showed that all four analytes were present in the cheese curd at concentrations that corresponded to 52- 65% of the initial quantity of each analyte added to milk.

The method was also successfully applied to quantify fenbendazole residues in Feta cheese made from incurred ovine milk. The results indicated that the only residues found in the cheese curd were oxendazole, fenbendazole sulphone and fenbendazole. The method was further used to quantify fenbendazole residues in yoghurt made from the above mentioned incurred ovine milk.

CONCLUSIONS

The developed method requires only a very small sample mass, and offers considerable savings in terms of solvent requirements, costly materials, sample manipulation, and analysis time. In addition, the method has satisfactory analytical characteristics with regard to recovery, sensitivity, selectivity, and repeatability. Sample throughput (extraction/clean-up/LC determination) is 15 samples in a total time of about 8 h by a single analyst. The method utilizes commercially available reagents and equipment and is designed to be performed safely by trained analysts. Owing to these advantages, the method might be considered suitable for the routine monitoring of fenbendazole residues in fermented dairy products.

REFERENCES