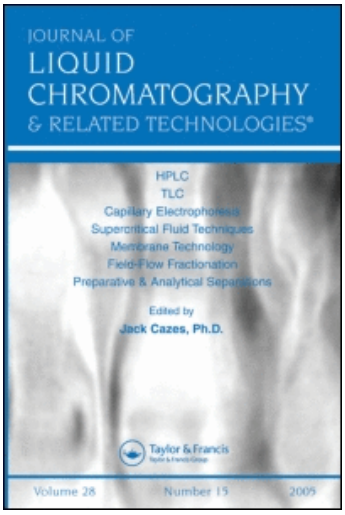

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DETERMINATION OF AMPROLIUM, ETHOPABATE, LASALOCID, MONENSIN, NARASIN, AND SALINOMYCIN IN FEED BY LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method for the determination of six coccidiostatics in feed is presented. Feed samples were homogenized with methanol–acetone–tetrahydrofuran. After addition of water, the samples were mixed and centrifuged. The compact bottom layer was re-extracted with methanol–water. After centrifugation, the combined supernatants were diluted and filtered through a Spin-X micro-centrifuge tube. Three different analytical columns were used. The calibration curves were linear in the investigated areas. The described assay offers a number of significant advantages compared to previously published methods for the detection and quantification of some coccidiostatics in feed. No derivatization is required.

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INTRODUCTION

The coccidiostatics amprolium (AMP), ethopabate (ETB), lasalocid (LAS), monensin (MON), narasin (NAR), and salinomycin (SAL) are frequently used in the prophylaxis and treatment of coccidiosis and leukocytozoonosis in poultry.[1–8] In addition, monensin and salinomycin are used as growth promoters in cattle and swine, respectively. Monensin, given to cows before calving, has also been found to increase milk production.[9]

Concern has been raised on the toxic/lethal effects of the misuse of these drugs,[10,11] which can occur when the drugs are present in feed not intended for a specific species or when given at higher levels than recommended. For these reasons, an accurate and precise method for the analysis of six coccidiostatics in both medicated feed, and possibly contaminated feed, is desirable.

In Norway, the concentration of AMP and ETB added to chicken feed ranges from 62.5 to 125 mg/kg for AMP and from 4 to 8 mg/kg for ETB. The concentration of LAS in chicken feed varies between 75 to 125 mg/kg, of MON between 90 to 125 mg/kg, of NAR between 60 to 70 mg/kg, and of SAL between 50 to 70 mg/kg.

Several methods based on fluorescence or UV-detection with post-column derivatization have been reported for analysis of the drugs.[3,4,6] These methods are, however, either time-consuming, require relatively large amounts of reagents, or cannot extract all six drugs simultaneously.

The purpose of the present study was to develop a rapid, simple, and specific LC-MS method for the determination of AMP, ETB, LAS, MON, NAR, and SAL in feed, with a sensitivity, which would at least meet requirements set by the Norwegian Feed Control Authority.

EXPERIMENTAL

Materials and Reagents

In the recovery experiments, samples of chicken, cattle, swine, and turkey drug-free feed produced by Felleskjøpet, Oslo, Norway, were used as control material and for spiking with, AMP, ETB, LAS, MON, NAR, and SAL.

All chemicals and solvents were of analytical or HPLC grade. Ethopabate was supplied by Merck Frosst Canada, (Pointe-Claire, Dorval, Quebec) and AMP, LAS, MON, NAR, and SAL by Sigma Co. (St. Louis, MO, USA). Stock solutions (1 mg/mL) and a mixed working standard (100 μg/mL) were prepared monthly by dilution with methanol and stored in a refrigerator at $+4^{\circ}$C. Spin-X micro-centrifuge tube filter (0.22 μm nylon) was supplied by Costar (USA).

Chromatographic Conditions

The analyses were performed on a Applied Biosystems LC-MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler (Perkin-Elmer). The acquired data were entered into a Model 8500 Apple Power Macintosh and processed with either Multiview 1.4 or MacQuant 1.6 software packages (Applied Biosystems), for spectral information and quantification data processing, respectively. An API 100 LC-MS system (Applied Biosystems) single quadruple mass spectrometer with a standard Turbo-Ion Spray Inlet for the API LC-MS System was employed for this study. The turbo probe of the instrument was maintained at 150°C and the probe air flowrate was 6 L/min. The turbo probe was not used for AMP and ETB. The LC-MS was set to collect multiple single-ion data in positive ion mode for the ions at m/z 243.3, 238, 613.5, 693.7, 787.5, and 773.6 for AMP, ETB, LAS, MON, NAR, and SAL, respectively. The entrance electrode voltages were adjusted to provide the optimum overall intensities for the six molecular ions.

A Merck column (Germany), Purospher STAR RP-18 endcapped (stainless steel, 55 × 4 mm I.D. packed with 3 μm particles), was employed to determine LAS, MON, NAR, and SAL. For ETB, the analytical column (stainless steel, 250 × 4.6 mm I.D.) was packed with 5 μm particles of Supelcosil LC-ABZ + Plus, while for AMP, the analytical column (150 × 4.6 μmm I.D.) was packed with 5 μm particles of Supelcosil LC-CN. The analytical columns were operated at 25°C. The respective guard columns for LC-ABZ + Plus, LC-CN and column STAR RP-18 were connected to an A-318 precolumn filter on line with an A-102X frits (Upchurch Scientific, USA). For LAS, MON, NAR, and SAL, the mobile phase consisted of 88% methanol and 12% of 0.1% formic acid in water (999 mL water + 1 mL formic acid.). The pump was operated isocratically at a flow rate of 1 mL/min.

For ETB, the mobile phase was 65% 10 mM ammonium acetate and 35% acetonitrile. The flow rate was 1 mL/min.

The mobile phase for AMP was 50% acetonitrile and 50% 10 mM ammonium acetate. The flow rate was 1 mL/min.

The LC eluent was split post-column approximately 1 : 20, so that c. 50 μL flowed into the Ion-Spray ion source.

Sample Pretreatment

Exactly 5 g of feed sample was weighed into a 50 mL graduated centrifuge tube with screw cap (Nunc, Roskilde, Denmark), and volumes of 10 mL methanol, or standard, and 10 mL acetone–tetrahydrofuran (6 + 4) were added. The mixture was homogenized for approximately 6 sec. in an Ultra-Turrax TP

18/10. After addition of 0.6 mL water, the mixture was blended, and left in an ultrasonic bath for 5 min. The sample was shaken for 3 sec and then centrifuged for 5 min (5000 rpm). The supernatant was transferred to another 50 mL graduated centrifuge tube. Ten milliliter of methanol-water (97 + 3) were added to the bottom compact layer. The mixture was shaken vigorously for approx. 10 sec. After centrifugation for 5 min, the supernatant was put into, and mixed with, the first supernatant. The volume was adjusted to 30 mL with methanol–water (90 + 10), mixed and then left in a refrigerator at +4°C for 30 min (sample A). To 100 μL of this mixture was added 5 mL of methanol–water (90 + 10). After blending, approximately 500 μL of the methanol-based sample was filtered through a Spin-X centrifuge tube by centrifugation for 3 min at 10,000 rpm (5600*g*). Aliquots of 10 μL were injected into the LC-MS at intervals of 8 min for the determination of LAS, MON, NAR, and SAL. To 100 μL sample A, 5 mL water was added and mixed. The mixture was also filtered through a Spin-X centrifuge filter. Aliquots of 10 and 30 μL were injected at intervals of 10 min for the determination of AMP and ETB, respectively.

Calibration Curves and Recovery Studies

The precision, recovery, and linearity for AMP, ETB, LAS, MON, NAR, and SAL were determined by spiking chicken feed samples with standard solutions to yield 20, 40, 75, 100, and 150 μg/g for AMP, LAS, MON, NAR, and SAL, respectively and 4, 6, 7.5, and 10 μg/g for ETB. Duplicate samples were used. The recovery rates were determined by comparing the analyses of spiked feed with those of standard solutions. The linearity of the standard curves for AMP, ETB, LAS, MON, NAR, and SAL in feed was calculated using peak height measurements.

To compare the analyses of spiked feed with those of standard solutions, the corresponding standards were diluted with methanol to 20 mL and 30 mL with methanol–water (90 + 10). Further dilution was performed as described for sample pretreatment. It was not necessary to filter these standard solutions through a Spin-X centrifuge tube.

RESULTS AND DISCUSSION

The standard curves were linear in the investigated areas from 20 to 150 μg/g for AMP, LAS, MON, NAR, and SAL, and from 4 to 10 μg/g for ETB in chicken feed. The correlation coefficients were $r = 0.999$ for all six drugs. The recovery and repeatabilities for AMP, ETB, LAS, MON, NAR, and SAL from feed are shown in Table 1.

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Table 1. Recovery and Repeatability for AMP, ETB, LAS, MON, NAR, and SAL from Spiked Samples of Chicken Feed

	AMP		ETB		LAS		MON		NAR		SAL	
Added[a]	40	150	4	10	40	150	40	150	40	150	40	150
No.[b]	8	8	8	8	8	8	8	8	8	8	8	8
S.D.[c]	1.0	0.6	1.0	1.0	2.6	1.1	1.0	1.3	1.3	1.8	0.5	0.3
Rec.[d]	96	98	92	87	92	91	98	98	95	98	98	99

[a]Concentration μg/g.
[b]No. of samples.
[c]Standard deviation.
[d]Recovery.

Chromatograms of cleaned samples from chicken feed and the corresponding samples spiked with AMP, ETB, LAS, MON, NAR, and SAL are shown in Figs. 1, 2, and 3.

Cattle, swine, and turkey feed showed a near similar baseline resolution compared with samples from chicken feed. The precision, recovery, and linearity of AMP, ETB, LAS, MON, NAR, and SAL in cattle, swine, and turkey feed were not validated in this study.

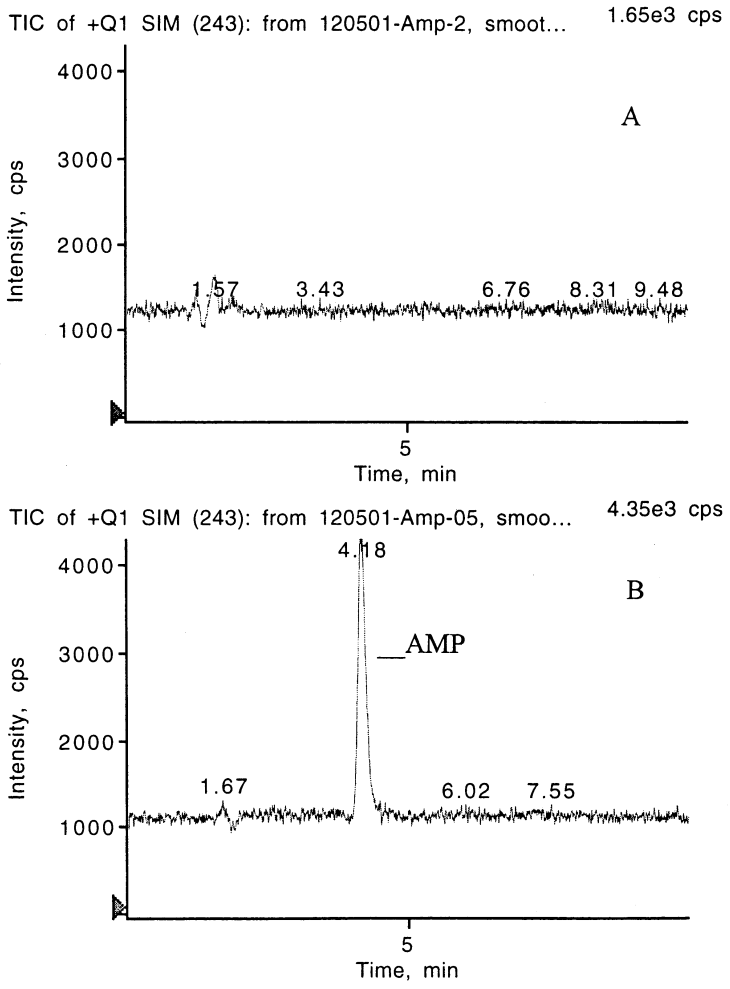

Figure 1. Chromatograms of extracts from chicken feed. A: drug-free feed, B: feed spiked with AMP (40 μg/g).

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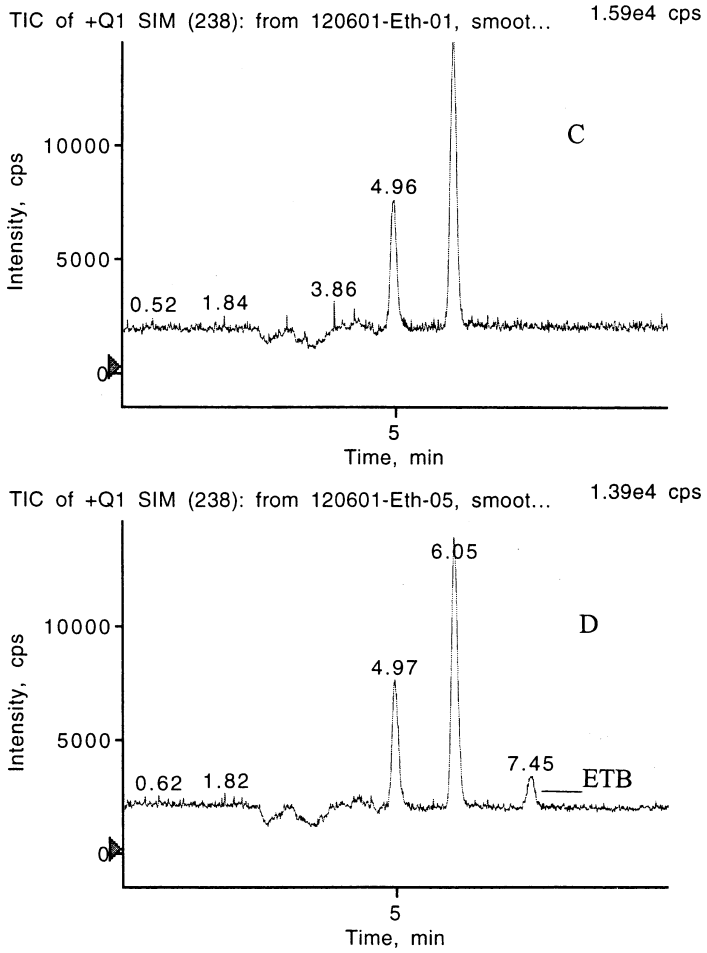

Figure 2. Chromatograms of extracts from chicken feed. C: drug-free feed, D: feed spiked with ETB (4 μg/g).

The limits of detection were close to 2 μg/g for AMP, LAS, MON, and NAR, 1.5 μg/g for ETB, and 2.5 μg/g for SAL in chicken feed. The limits of quantification were 4 μg/g for AMP, LAS, MON, and NAR, 3 μg/g for ETB, and 5 μg/g for SAL.

The detection limits of the assays were calculated to be three times the baseline noise from drug-free feed. No interference was seen during analysis, when calibrating the curves, or when performing recovery studies.

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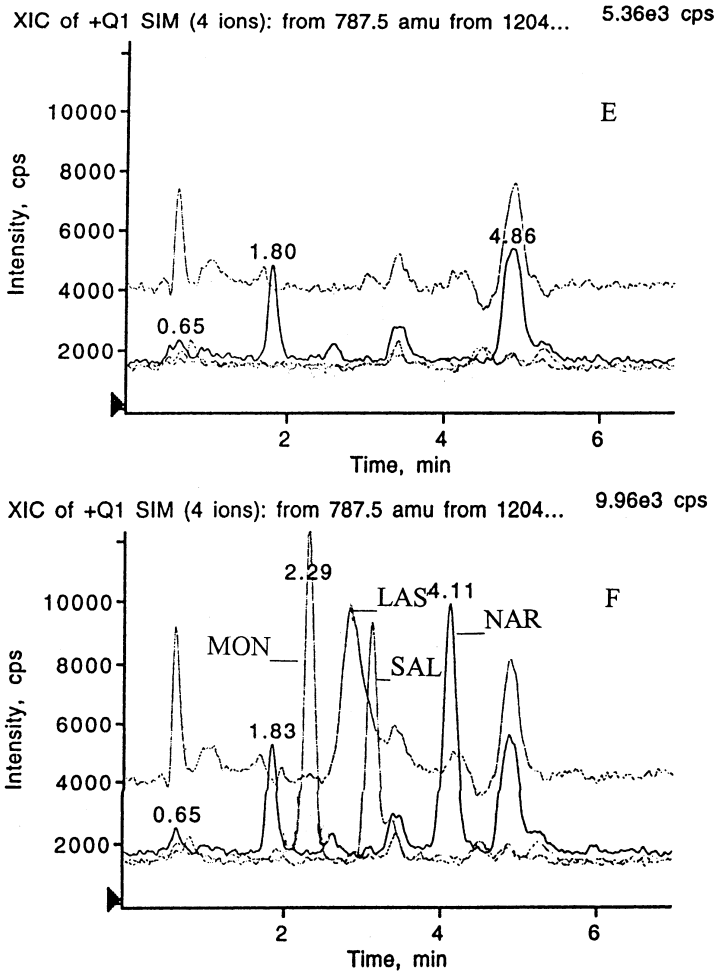

Figure 3. Chromatograms of extracts from chicken feed. E: drug free feed, F: feed spiked with LAS, MON, NAR, and SAL (40 μg/g).

When a new Lichrocart Purospher Star RP 18 column was taken into use, the column was flushed with 100% acetonitrile for 10 min at a flow rate of 0.6 mL/min and at a flow rate of 0.9 mL/min for 40 min, before equilibrating with the mobile phase. The Star 55×4 mm column does not need a guard column.

The described assay offers a number of significant advantages compared to previously published methods for the detection and quantification of AMP, ETB, LAS, MON, NAR, and SAL in feed. The detection limit is good, and no derivatization is required.

The method presented is selective, robust, and accurate. The method is used in the routine control of feed for the Norwegian Feed Control Authority.

The advantage of the LC-MS technique lies in the combination of the separation capabilities of HPLC and the power of MS as an identification and confirmation method with high sesitivity, selectivity, and quantitative capability. While conventional HPLC methods may require long complex separations, the LC-MS method generally requires only a simple clean-up procedure. Thus, LC-MS seems to provide a better alternative than HPLC.

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