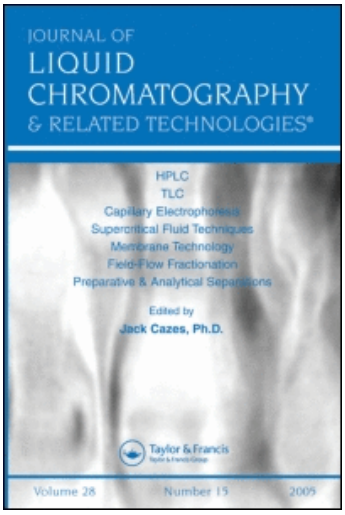


**Journal of Liquid Chromatography & Related Technologies**
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# DETERMINATION OF AMPROLIUM, ETHOPABATE, LASALOCID, MONENSIN, NARASIN, AND SALINOMYCIN IN CHICKEN TISSUES, PLASMA, AND EGG USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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Online publication date: 15 April 2001

J. LIQ. CHROM. & REL. TECHNOL., 23(10), 1585–1598 (2000)

# DETERMINATION OF AMPROLIUM, ETHOPABATE, LASALOCID, MONENSIN, NARASIN, AND SALINOMYCIN IN CHICKEN TISSUES, PLASMA, AND EGG USING LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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## ABSTRACT

A liquid chromatographic-atmospheric pressure ionization ion spray method for the determination of some coccidiostatics in chicken egg, fat, liver, muscle, and plasma is presented. The samples were extracted with acetone-tetrahydrofuran. The water and the organic layer was then separated using a liquid-liquid extraction step and the organic phase was then evaporated to dryness. The dry residue was diluted in hexane and a portion of the organic phase was cleaned-up with a simple step for ethopabate analyses while the rest of the organic phase was purified using a silica solid phase extraction column to analyse lasalocid, monensin, narasin, and salinomycin. The water phase (amprolium analyses) was diluted with water, filtered, and injected into the LC-MS. Three different analytical columns were used. The detection limits were from 1 ng/g to 7 ng/g for chicken tissue and from 4 to 10 ng/mL for plasma.

## INTRODUCTION

The ionophore 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 chicken.[1,15] The residues of these drugs may represent a potential health risk to consumers.

Several analytical methods based on colorimetry,[2] gas chromatography,[3-5] and thin-layer chromatography-bioautography[6] have been published for the determination of one or more of these compounds in different biological materials. Most confirmatory methods published use high performance liquid chromatography (HPLC) with fluorescence and UV detection. Since the ionophores do not possess any significant UV absorbance, derivatisation is generally required.[7-13] The different methods are, however, time consuming and require the use of large quantities of chemical reagents.



More recently, a method for the determination of lasalocid, and a method for the joint determination of monensin, salinomycin, and narasin, using liquid chromatography-electrospray mass spectromery, have been published.[14,15]

However, none of the published methods appeared to be applicable for the determination of all ionophore coccidiostatics, simultaneously from the same sample.

The purpose of the present study was to develop a time-saving, cost-effective, and sensitive method, for the determination of AMP, ETB, LAS, MON, NAR, and SAL, which required only small quantities of chemical reagents.

## EXPERIMENTAL

### Materials and Reagents

Samples of egg (E), fat (F), liver (L), meat (M) and plasma (P) from chickens were used.

All chemicals and solvents were of analytical or HPLC grade. ETB was supplied by Merck Frosst Canada, Pointe - Claire, Dorval, Quebec. AMP, LAS, MON, NAR, and SAL were supplied by Sigma Co. (St. Louis, MO, USA). Stock solutions (1mg/mL) and working standards (1 and 0.1 μg/mL) were prepared by dilution with methanol and stored in a refrigerator at + 4°C. Extraction columns Sep-pak Vac RC (500 mg) silica cartridges for solid phase extraction (SPE) were purchased from Waters (Waters Corporation, Milford, Massachusett

USA.). Spin-X micro-centrifuge tube filter (0.22 μm nylon) was supplied by Costar (USA).

Solution A was a mixture of 70% methanol and 30% 0.022 M 1-heptane sulfonic acid sodium salt (Supelco USA) - 0.01 M di-sodium hydrogenphosphate-2-hydrate (Ferax, Germany). The solution was made by dissolving 4.45 g/L heptane sulfonate and 1.8 g/L di-sodium hydrogenphosphate 2-hydrate in c. 750 mL of water when preparing 1 litre of solution. The pH was then adjusted to c. 6.3 with 5 M H3PO4 and to 6.0 with 1 M H3PO4, and the solution made up to volume (1 L) with water, whereafter, the pH again was adjusted to 6.0 with 1 M H3PO4.

Solution B, consisting of 0.15% trichloroacetic acid (TCA) in acetone, was prepared by first dissolving 85g TCA in 15 g water to obtain a stock solution of 85% TCA in water. The stock solution was stored in a refrigerator (+ 4°C). Solution B was then prepared by diluting 150 μL stock solution with acetone to 100 mL.



**Chromatographic Conditions**

The analyses were performed on a Perkin-Elmer LC-MS system, consisting of a Series 200 quaternary pump and a Series 200 autosampler. The acquired data were entered into a Model 8500 Apple Power Macintosh and processed with either Multiview 1.4 or MacQuan 1.6 software packages (Perkin-Elmer), for spectral information and quantification data processing, respectively. An API 100 LC-MS system (PE SCIEX) 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 flow-rates of air for the probe 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, 238.2, 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 Supelco Discovery C18 column (stainless steel, 250 x 4.6 mm I. D. packed with 5 μm particles) was employed for determining LAS, MON, NAR, and SAL. For ETB, the analytical column (stainless steel, 250 x 4.6 mm I.D.) was packed with 5 μm particles of Supelcosil LC-ABZ + Plus, while for AMP the analytical column (150 x 4.6 mm I.D.) was packed with 5 μm particles of Supelcosil LC-CN. The respective guard columns 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 85% methanol and

15% 10 mM (0.77 g/L) ammonium acetate. The flow rate was 0.8 mL/min for 15 min followed by 1 mL/min for 10 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. The samples were injected at intervals of 10 min for the determination of AMP and ETB and at intervals of 25 min. for LAS, MON, NAR, and SAL.

**Sample Pretreatment**



The stepwise procedure for pretreatment of egg, fat, liver, and meat samples is shown in Figure 1.

Volumes of 0.5 mL methanol or standard (the total volume added in this step should be 0.5 mL), 0.5 mL water and 6 mL acetone-tetrahydrofuran (6 + 4) were added to 3 g of sample. The mixture was homogenized for approximately 6 sec. in an Ultra-Turrax TP 18/10 (Janke & Junkel KG, Ika Werk, Staufen, Germany) and left in an ultrasonic bath for 5 min. After centrifugation for approximately 5 min. (5000 rpm), a 5 mL volume of the supernatant (corresponding to 1.5 g for E, L, and M), or a 2.5 mL volume (from the upper layer for F) corresponding to 0.75 g for F, was pipetted into a conical centrifuge tube, and 6 mL diethyl ether-hexane (6 + 4) was added. For F, 0.5 mL water was also added. The mixture was shaken vigorously for approx. 5 sec. After centrifugation for 3 min (3500 rpm), the upper layer (organic phase) was transferred to another glass-stoppered tube; the bottom water layer being retained for subsequent analysis of AMP (see Section I below). The upper layer was mixed with 5 mL hexane, and centrifuged for 3 min. The supernatant was transferred into a glass-stoppered tube and evaporated to dryness under a stream of air, using a Reacti-Term heating module at 60°C and a Reacti Vap evaporating unit (Pierce, Rockford IL, USA). Waiting until the sample had achieved room temperature, the dry residue was dissolved in 1.5 mL hexane, ultrasonicated for 5 min. (7 min. for L), and mixed. Hexane (0.5 mL) corresponding to 0.5 g samples for E, L, and M, and 0.25 g samples for F, was transferred into another glass-stoppered tube for ETB analysis (Section II). The remaining 1 mL hexane sample (Section III), corresponding to 1g for E, L, and M, and 0.5 g for samples from F, was loaded into a conditioned SI column.

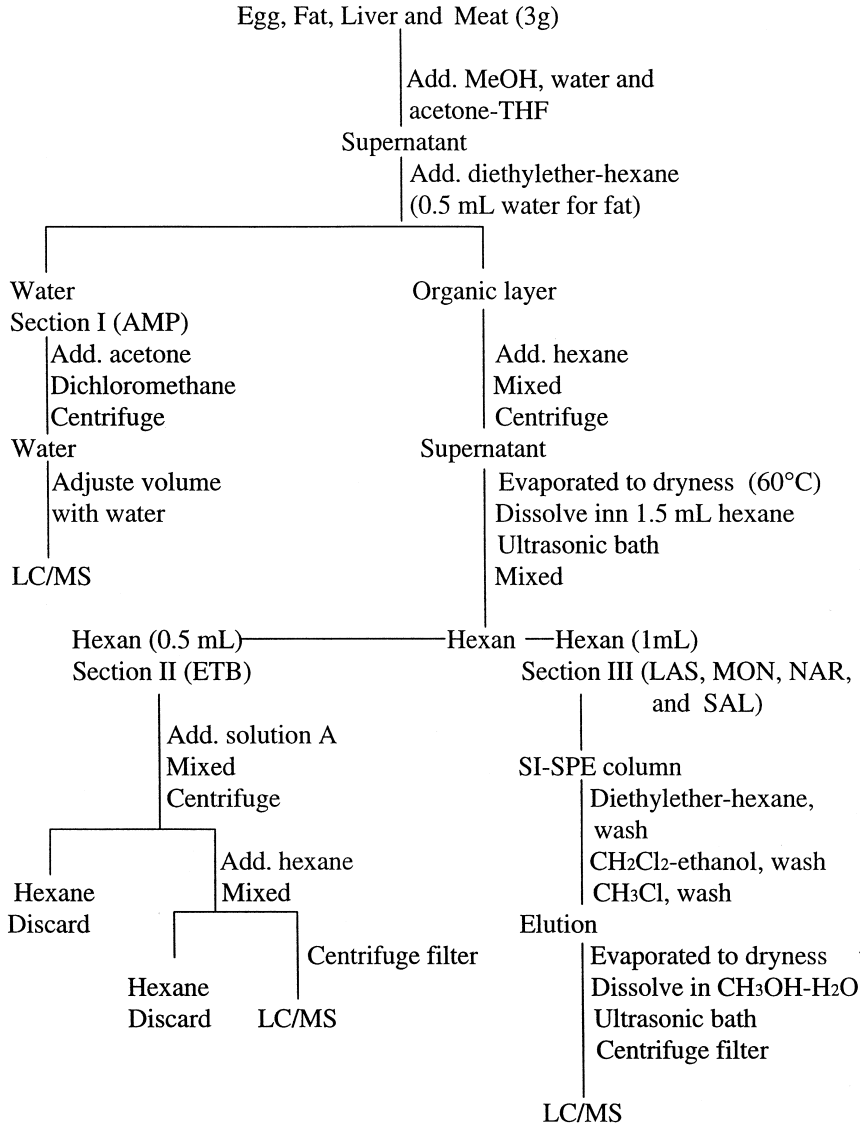


**Figure 1**. Extraction and Clean-up Procedure for AMP, ETB, LAS, MON, NAR and SAL from Chicken Egg, Fat, Liver and Meat.

***Section I (AMP)***

One mL acetone and 5 mL $CH_2Cl_2$ were added to the water based sample (see sample pretreatment). The mixture was shaken vigorously for 10 sec., and centrifuged for 3 min. The upper layer (water) was transferred to a graduate glass-stoppered tube. The volume was adjusted to 2 mL with water for E, L, and M samples, and to 1 mL for F, corresponding to 1.5 g samples for E, L, and M, and to a 0.75 g sample for F. Approximately 500 µL of the water-based sample was filtered through a Spin-X centrifuge tube by centrifugation for 2 min. at 10000 rpm. (5600g). Aliquots of 80 µL were injected into the LC-MS at intervals of 10 min. for the determination of AMP.

***Section II (ETB)***

To the 0.5 mL hexane sample (see sample pretreatment), 400 µL (500 L for liver) of solution A was added, vortex-mixed vigorously for 30 sec., followed by centrifugation for 3 min. The hexane layer was discharged and 0.5 mL hexane was added. After the sample was mixed for 6 sec., and centrifuged for 3 min., the hexane layer was discharged. The methanol based phase was filtered through a Spin-X filter. Aliquots of 30 µL were injected into the LC-MS at intervals of 10 min. for the determination of ETB.



***Section III (LAS, MON, NAR, and SAL)***

*Clean-Up on SPE-Column*

The SI column was conditioned with 5 mL hexane, and the sample extract was loaded into the column (see sample pretreatment). Thereafter, the glass-stoppered tube was rinsed with 1 mL hexane which was also loaded into the column. The column was washed with 8 mL diethyl ether-hexane (6 + 4), 8 mL dichloromethane-ethanol (96%) (99.5 + 0.5), and 8 mL chloroform. Application of the sample, and washing of the SPE column all took place under gravity flow (dropwise). Afterwards the SPE column was suctioned to dryness for c. 10 sec. (at a vacuum of -10 inches Hg, using a Vac Master system from International Sorbent Technology). The column was then eluated with 3 x 1 mL $CH_2Cl_2$-$CH_3OH$ (90 + 10), with a vacuum of -5 inches Hg. The eluate was collected and evaporated to dryness. To the dry residue, 0.4 mL (0.5 mL for liver) of $CH_3OH$-$H_2O$ (8 + 2) was added, mixed with a whirlimixer for 5 sec., left in an ultrasonic bath for 5 min to reconstitute the residue, and then filtered through a Spin-X centrifuge filter.

Aliquots of 30 µL (20 µL for liver) were injected into the LC-MS at intervals of 25 min. for the determination of LAS, MON, NAR, and SAL.

*Plasma*

Volumes of 100 µL methanol-water (8 + 2) or standard, and 400 µL solution B, were added to 500 µL plasma samples. The mixture was vortex-mixed

and centrifuged (4 min. 4000 rpm). The supernatant was filtered through a Spin- X centrifuge filter. Aliquots of 30 µL were injected into the LC-MS for the determination of LAS, MON, NAR, and SAL. For the AMP and ETB analyses, 100 µL filtrated and 100 µL water were mixed. Aliquots of 80 and 30 µL were injected into the LC-MS 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 egg, fat, liver, and meat samples with standard solutions to yield 5, 10, 15, 20, 50, 100, and 150 ng/g, and for plasma to yield 10, 20, 30, 50, 100, 200, 500, 1000, and 2000 ng/mL, respectively. Duplicate samples were used. The recovery rates were determined by comparing analysis of spiked egg, fat, liver, meat, and skin, with those of standard solutions. The linearity of the standard curves for AMP, ETB, LAS, MON, NAR, and SAL in E, F, L, M, and P were calculated using peak height measurements.



## RESULTS AND DISCUSSION

The standard curves were linear in the investigated areas from 5 to 150 ng/g for AMP, ETB, LAS, MON, NAR, and SAL for egg, fat, liver, and meat. From 20 to 2000 ng/mL for AMP, 15 to 2000 ng/mL for ETB and LAS, and 10 to 2000 ng/mL for MON, NAR, and SAL in plasma. The corresponding correlation coefficients are shown in Table 1 and the recovery and repeatabilities for AMP, ETB, LAS, MON, NAR, and SAL from egg, fat, liver, and meat are shown in Table 2 and for plasma in Table 3.

In many laboratories, a stream of nitrogen is used to evaporate AMP, ETB, LAS, MON, NAR, and SAL samples to dryness. We compared nitrogen against air produced from a central air compressor (generator) for evaporating the sam-

**Table 1**

**Correlation Coefficients for AMP from ETB, LAS, MON, NAR, and SAL from E, F, L, M, and P**

| | AMP | ETB | LAS | MON | NAR | SAL |
|---|---|---|---|---|---|---|
| E | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 |
| F | 0.998 | 0.999 | 0.998 | 0.999 | 0.999 | 0.999 |
| L | 0.998 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 |
| M | 0.998 | 0.999 | 0.998 | 0.998 | 0.999 | 0.999 |
| P | 0.998 | 0.999 | 0.999 | 0.999 | 0.999 | 0.999 |

**Table 2**

**Recovery and Repeatability for AMP, ETB, LAS, MON, NAR, and SAL from Spiked Samples of Egg, Fat, Liver, and Meat**

| | AMP | | ETB | | LAS | | MON | | NAR | | SAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Added[a] | 20 | 50 | 20 | 50 | 20 | 50 | 20 | 50 | 20 | 50 | 20 | 50 |
| No.[b] | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| **Egg** | | | | | | | | | | | | |
| S.D.%[c] | 2.3 | 3.9 | 1.4 | 1.2 | 2.2 | 1.6 | 6.2 | 3.0 | 3.1 | 2.4 | 3.8 | 3.9 |
| Rec.%[d] | 100 | 100 | 84 | 86 | 75 | 72 | 94 | 96 | 100 | 96 | 89 | 89 |
| **Fat** | | | | | | | | | | | | |
| S.D. | 0.8 | 1.2 | 1.5 | 2.7 | 2.5 | 1.9 | 1.4 | 4.2 | 1.8 | 3.5 | 2.8 | 4.3 |
| Rec. | 181 | 182 | 100 | 112 | 85 | 77 | 114 | 121 | 86 | 93 | 102 | 106 |
| **Liver** | | | | | | | | | | | | |
| S.D. | 1.2 | 1.9 | 4.2 | 2.2 | 1.3 | 0.6 | 1.4 | 3.4 | 2.0 | 3.3 | 3.2 | 3.6 |
| Rec. | 98 | 102 | 106 | 96 | 62 | 61 | 85 | 92 | 97 | 103 | 92 | 93 |
| **Meat** | | | | | | | | | | | | |
| S.D. | 6.8 | 3.4 | 1.4 | 1.2 | 2.3 | 1.7 | 1.7 | 2.0 | 3.0 | 2.5 | 3.7 | 3.8 |
| Rec. | 92 | 88 | 84 | 86 | 76 | 74 | 95 | 101 | 100 | 97 | 90 | 90 |

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



ples of AMP, ETB, LAS, MON, NAR, and SAL from chicken tissues, egg, and plasma. No differences were found.

AMP has affinity for water. The high recovery of AMP from fat is a consequence of the presence of the small quantity of water in fat which concen-

**Table 3**

**Recovery and Repeatability for AMP, ETB, LAS, MON, NAR, SAL, from Spiked Samples of 0.5 mL Plasma**

| | AMP | | ETB | | LAS | | MON | | NAR | | SAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Added[a] | 100 | 1000 | 100 | 1000 | 100 | 1000 | 100 | 1000 | 100 | 1000 | 100 | 1000 |
| No.[b] | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| S.D.%[c] | 2.9 | 1.8 | 1.2 | 0.6 | 1.2 | 1.2 | 0.7 | 1.4 | 1.2 | 1.2 | 1.3 | 0.5 |
| Rec.%[d] | 120 | 125 | 94 | 97 | 95 | 99 | 96 | 99 | 96 | 98 | 97 | 99 |

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

trates under the extraction phase principally in the upper-layer (see sample pretreatment) which is used for analysis. This does not affect the result for a real sample, because the sample result is calculated from data from a spiked fat standard curve.

Chromatograms of cleaned samples from meat and the corresponding samples spiked with AMP, ETB, LAS, MON, NAR, and SAL are shown in Figures 2, 3, and 4.

Egg, fat, liver, and plasma show a near similar baseline resolution to samples from meat.

For AMP and ETB in plasma, the sample was mixed with water (1 + 1) because otherwise the high percentage of organic chemicals in these samples will change the baseline resolution for AMP and ETB.[16]



The detection limit of the assay depends mainly on the sensitivity of the LC-MS. This in turn could be influenced by such factors as the position of the ion spray inlet, the composition of the mobile phase, and the flow-rate of the mobile phase into the ion source.

The chromatographic system appeared to be efficient for the determination of the six ionophores in egg, fat, liver, meat, and plasma. The limit of quantification and the limits of determination are shown in Table 4.

The detection limit of the assay was calculated to be three time the baseline noise from-free tissue. No interference was seen during analysis, (with the exception of SAL in liver which had a small interference) when calibrating the curves, or when performing recovery studies.

The method presented in this paper is selective, robust, sensitive, and accurate.

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 tissues and egg. The detection limit is good. The extraction procedure is simple but effective. No derivatization is required and only one extraction is necessary.

The conformity of the graduation of the glass centrifuge tube to the end volume of AMP was controlled beforehand.

Under the sample pretreatment acetone-THF, i.e. was added; automate-pipette with tips gave a irregular volume. This problem was avoided with a bottle top dispenser, which gave good results.

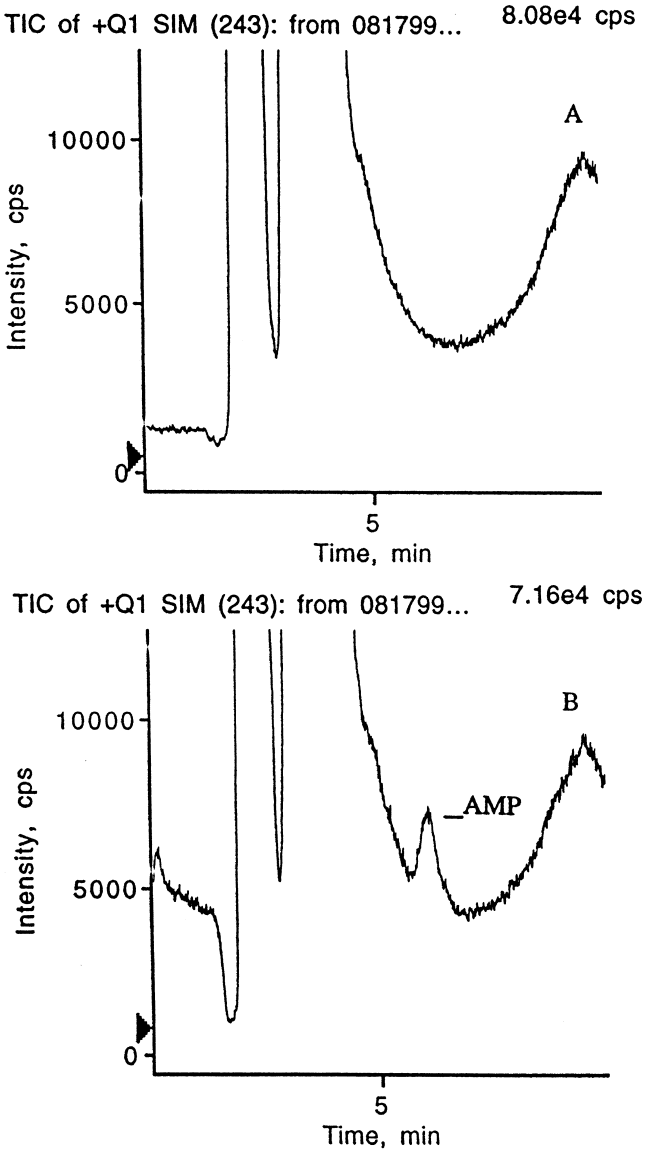


**Figure 2**. Chromatograms of extracts from chicken meat. A: drug-free meat, B: meat spiked with AMP (10 ng/g).

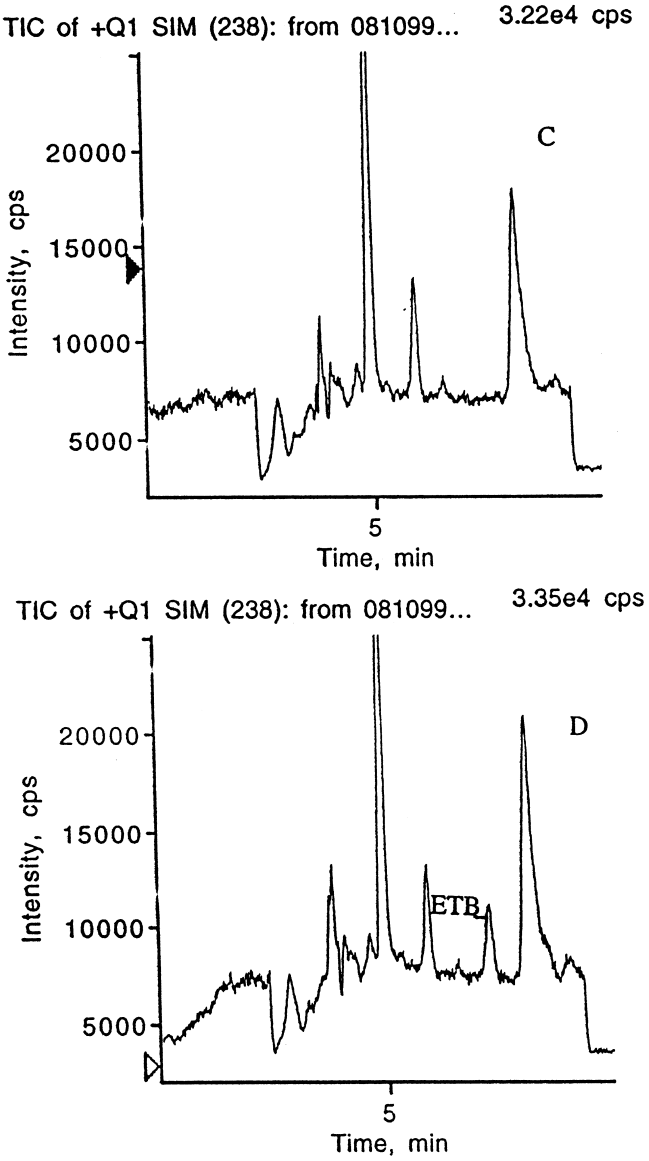


**Figure 3**. Chromatograms of extracts from chicken meat. C: drug-free meat, D: meat spiked with ETB (10 ng/g).

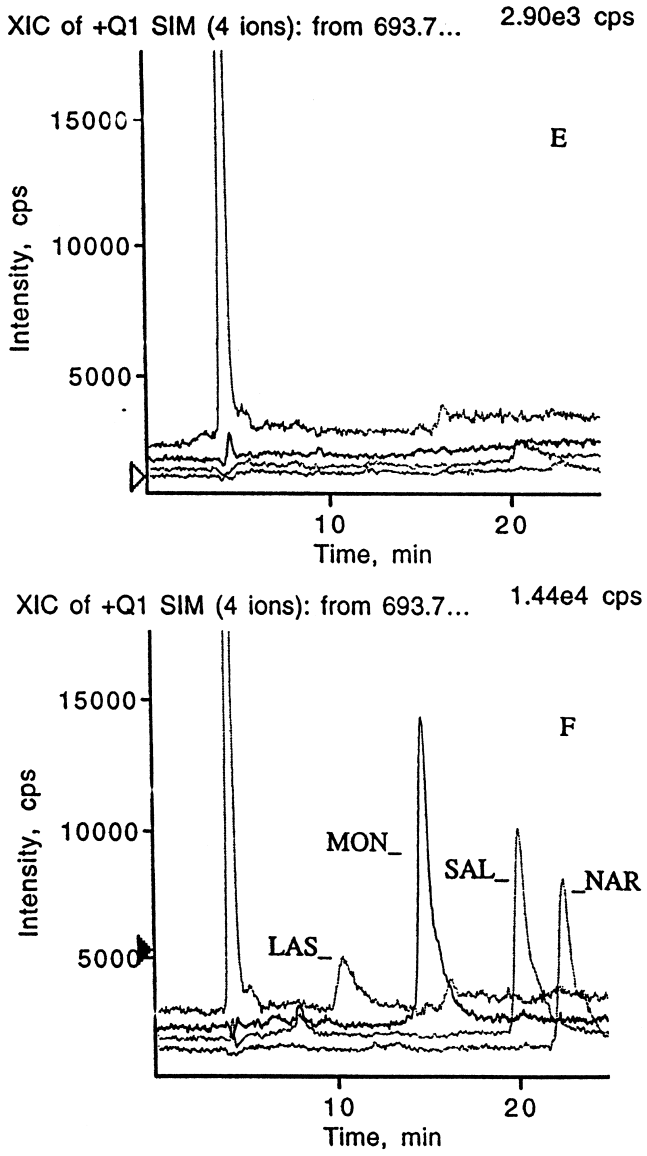


**Figure 4**. Chromatograms of extracts from chicken meat. E: drug-free meat, F: meat spiked with LAS, MON, NAR and SAL. (10 ng/g).

**Table 4**

**Determination and Quantification Limits for AMP, ETB, LAS, MON, NAR, and SAL in Samples of Egg, Fat, Liver, Meat, and Plasma**

| | AMP | | ETB | | LAS | | MON | | NAR | | SAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | D.[a] | Q.[b] | D. | Q. | D. | Q. | D. | Q. | D. | Q. | D. | Q. |
| E | 2 | 4 | 3 | 6 | 3 | 6 | 1 | 2 | 5 | 10 | 5 | 10 |
| F | 1 | 2 | 2 | 4 | 3 | 6 | 1 | 3 | 3 | 6 | 3 | 6 |
| L | 2 | 4 | 2 | 4 | 7 | 15 | 2 | 4 | 3 | 6 | 5 | 10 |
| M | 2 | 4 | 2 | 4 | 3 | 6 | 1 | 2 | 2 | 4 | 3 | 6 |
| P[c] | 7 | 15 | 7 | 15 | 7 | 15 | 4 | 8 | 5 | 10 | 10 | 20 |

[a] Determination limit (ng/g). [b] Quantification limit (ng/g). [c] In ng/mL.



It is also important to follow the recommendations of the producer to store Supelcosil LC-ABZ+Pus, LC-CN and C18 Discovery column, to avoid retention loss.

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 sensitivity, selectivity, and quantitative capability. Quantification using selected ion monitoring has high selectivity, sensitivity, and broad dynamic range. 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.

## ACKNOWLEDGMENT

We are grateful to the Norwegian Research Council for financial support.

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Received September 4, 1999 Author's Revisions January 29, 2000
Accepted December 5, 1999 Manuscript 5165