A New Approach to the Simultaneous Analysis of Underivatized Ionophoric Antibiotics using Liquid Chromatography with Charged Aerosol Detection

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Overview

Purpose: To develop an analytical method that can be used to determine ionophoric antibiotics in food without post-column derivatization.

Methods: Four ionophoric antibiotics were chromatographed and resolved using the Thermo Scientific Acclaim RSLC C18 column on the Thermo Scientific Dionex UltiMate 3000 RSLC and the Thermo Scientific Dionex Corona ultra RS charged aerosol detector, without derivatization.

Results: This sensitive method can determine low amounts of ionophores in samples, and provides the greatest resolution value between monensin and narasin of any method found in the literature. Two food samples, egg and chicken, were processed and analyzed for possible matrix interference.

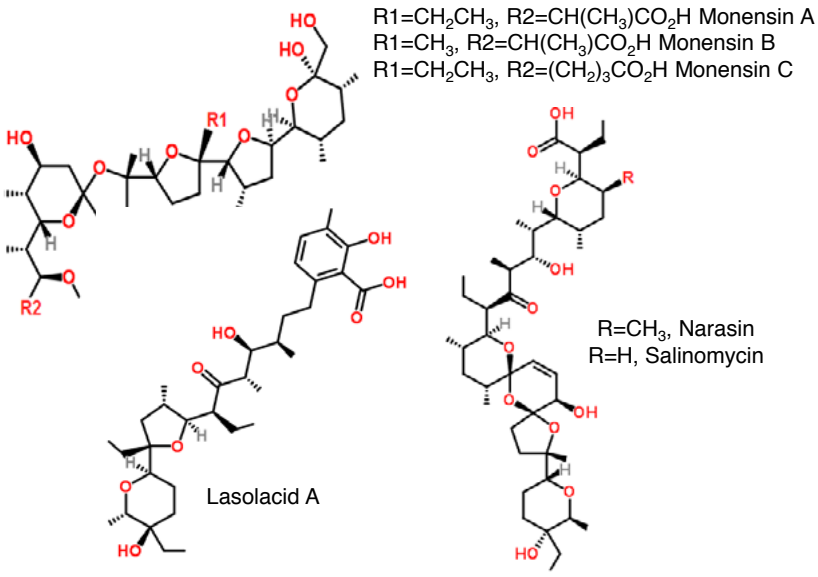
Introduction

Antibiotics can be categorized based on their chemical structure, including: beta-lactams, aminoglycosides, tetracyclines, fluoroquinolines, macrolides, and polyether ionophores. The use of polyether ionophorous antibiotics (monensin, salinomycin, lasolacid A, and narasin) in industrial agriculture is significant, with over 3,700,000 kg approved for use in 2009 by the Food & Drug Administration.[1] Ionophores are approved feed additives, and are used to a large extent in the poultry and beef production industry to control and prevent coccidiosis disease. Adding these ionophores to animal feeds can improve growth rates, but there is concern that antibiotic residues may remain in tissues, food products, and the environment. Some animals such as horses,[2] certain avian species,[3,4] dogs,[5] and cats[6] are especially sensitive to ionophore toxicity. Thus, there is a need for sensitive methods for their analysis.

These antibiotics originate from natural sources and possess a weak chromophore, which make them difficult to analyze. Current HPLC-UV methods require the use of post-column derivatization with a methanolic, sulphuric acid vanillin reagent and heating to form a derivative that has a UV response. This not only increases system complexity but also reduces the peak resolution and sensitivity.[7] Newer analytical methods for multi-residue analysis using mass spectrometry have been developed but require more highly-skilled operators than for other methods using ultraviolet or charged aerosol detection.

A sensitive HPLC charged aerosol detection method was developed for the simultaneous detection of underivatized ionophoric coccidiostats, including narasin sodium ("narasin"), monensin ("monensin"), lasolacid A, and salinomycin sodium ("salinomycin") (Figure 1). The use of the highly selective 2.2 µm C18 RSLC column with the Corona™ ultra RS™ charged aerosol detector (CAD™) enabled the measurement of these analytes to low ng on-column sensitivity while satisfying the USP system suitability requirements. Chromatographic profiles of food samples (chicken and egg) are shown to illustrate matrix effects.

FIGURE 1. Structures of six ionophoric analytes.



Methods

Sample Preparation

The process followed that outlined in Rosén *et al (*2001)[8] and briefly described here: 5 g of blended sample was homogenized in 15 mL methanol/water (87:13). The mixture was centrifuged for five minutes at 5000 g. The clarified supernatant was then processed through a 200 mg C18 solid phase extraction (SPE) column using the following steps: 4 mL of 100% methanol and 2 mL of water (column conditioning). Aliquots of 9 mL of samples were passed through the SPE, followed by 6 mL of 80% methanol (column wash). Finally, the analytes were eluted using 6 mL of 91% methanol.

Liquid Chromatography

HPLC System: UltiMate™ 3000 RSLC DGP
HPLC Column: Thermo Scientific Acclaim RSLC 120 C18, 2.2 µm, 2.1 × 250 mm
Column Temp.: 50 °C
Mobile Phase A: water/methanol (350:650), 0.1% acetic acid
B: methanol, 0.1% acetic acid
Flow Rate: 0.50 – 0.85 mL/min
Gradient:

Time (min)	Flow Rate (mL/min)	%A	%B
-3.0	0.55	100.0	0.0
0.0	0.50	100.0	0.0
0.5	0.50	100.0	0.0
1.0	0.50	58.0	42.0
2.0	0.51	56.5	43.5
4.0	0.56	50.0	50.0
10.0	0.56	45.0	55.0
23.0	0.65	45.0	55.0
26.0	0.65	37.0	63.0
28.0	0.65	0.0	100.0
33.0	0.85	0.0	100.0
33.0	0.85	100.0	0.0
35.0	0.55	100.0	0.0

Sample Solvent: water/methanol (9:91)
Sample Temperature: 15 °C
Injection Volume: 10 µL
Detector: Corona ultra RS
Nebulizer Temperature: 15 °C
Filter Setting: None
Analysis Time: 38 minutes.

Data Analysis

The HPLC system, data collection and processing were all operated by and performed on the Thermo Scientific Dionex Chromeleon 7.1 SR 1 software.

Results

System Suitability

A sample containing monensin sodium and narasin at a concentration of 400 µg/mL (4000 ng on-column (o.c.)) was analyzed in triplicate, and overlaid chromatograms are presented in Figure 2. The system suitability requirements specified in the USP method were easily exceeded, including peak symmetry for monensin A (Table 1).

Calibration

Aliquots of 125 µL of each stock standard solution of monensin sodium, (1 mg/mL in methanol), salinomycin (1 mg/mL in methanol), narasin sodium (1 mg/mL in methanol), and 250 µL of lasolacid A (0.1 mg/mL in acetone) were mixed together and then diluted with 375 µL water (1:1). Standards were diluted sequentially with water/methanol (1:1), and analyzed in triplicate. Peak areas for each analyte were plotted against the amount injected and the data were fit to inverted second-polynomials to obtain calibration curves for the whole range of amounts (Figure 3). Data were fit to linear equations for amounts < 315 ng o.c. for all but the lasolacid A, as shown in Figure 4. Correlations for both calibration ranges were high, with coefficients, $r^2 > 0.9999$ for all four analytes.

Instrument precision varied from 0.1 to 6 peak area %RSD for monensin, salinomycin, and narasin (9.8 to 1250 ng o.c.), and from 0.5 to 12 peak area %RSD for lasolacid A (2 to 250 ng o.c.). Overlaid chromatograms are shown in Figure 5.

FIGURE 2. System suitability chromatogram with monensin sodium and narasin sodium at 4000 ng o.c.

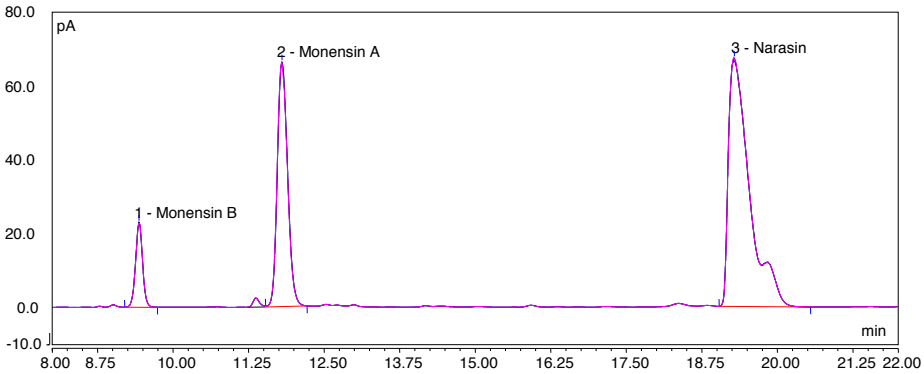
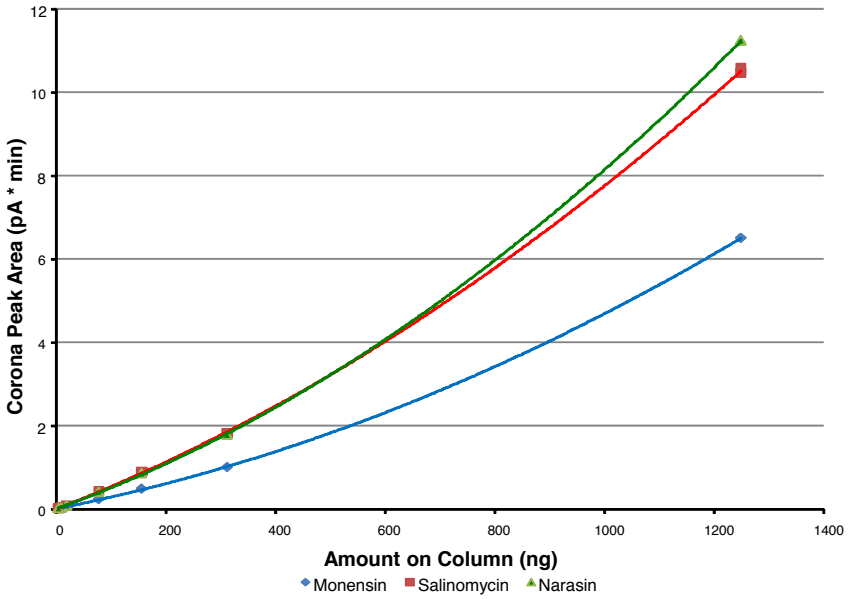


Table 1. System suitability results compared to USP requirements.

Parameter	USP Specification	Method Result	Pass / Fail
Rs (Monensin A,B)	NLT 1.25	9.2	Pass
Rs (Monensin A, Narasin)	NLT 3.5	16.6	Pass
Tf (Monensin A)	NMT 1.4	1.2	Pass
%RSD, 4000 ng o.c.	NMT 2.0	0.1	Pass

FIGURE 3. Calibration curves for monensin, salinomycin, and narasin from 4.9–1250 ng o.c., fit to a second-order polynomial.



Sensitivity

Limits of detection (LOD) and quantitation (LOQ) were determined using signal-to-noise values of 3.3 and 10.0, respectively, based on the 78 ng o.c. peak areas. LOD values were below 10 ng o.c., and LOQ values were at or below 31 ng o.c., as summarized in Table 2. The per mass of sample sensitivity, using the sample preparation above and assuming the 90% recovery value cited in literature[8], is 200 ppb (w/w). The SPE preparation used here was not optimized. Additional sample preparation (optimized SPE, and perhaps larger sample mass) and larger injection volumes may enhance the sensitivity further in order to obtain the 50 ppb limits required by U.S. MRL database.[9]

FIGURE 4. Low level calibration curves for monensin, salinomycin, and narasin fit to linear equations, from 4.9 to 313 ng o.c. lasolacid A, fit using second-order polynomial from 0.5 to 250 ng o.c.

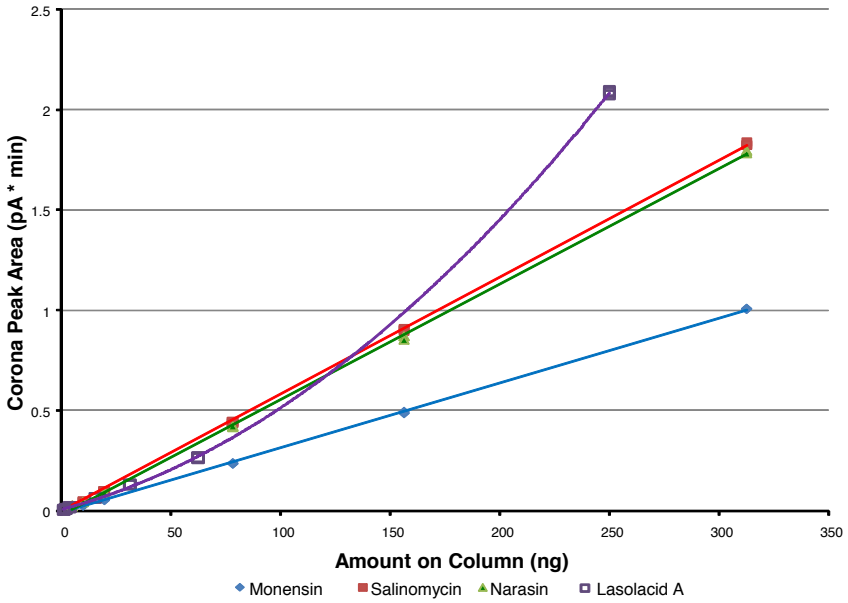


Table 2. Chromatography limits of detection (LOD) and quantitation (LOQ)

Analyte	LOD (ng o.c.)	LOQ (ng o.c.)
Monensin A sodium	8	27
Lasolacid A	2	7
Salinomycin sodium	7	22
Narasin sodium	9	31

Sample Results

Samples of eggs and chicken meat were homogenized and two 5 g samples of each were added to different 25 mL centrifuge tubes. To one of each pair, 500 ng of salinomycin was added as a spike. Even though there was loss of the spiked analyte, possibly due to a different SPE column being used than reported in literature, there was sufficient sensitivity and chromatographic resolution to verify the presence of salinomycin in the spiked sample (Figure 5).

The salinomycin peak elutes between other peaks that are found in the matrix. To see whether additional matrix components interfered with the measurement of the other ionophores, chromatograms for the standards and an extracted and processed chicken sample were compared (Figure 6).

FIGURE 5. HPLC chromatograms of egg sample SPE eluent (91% methanol) unspiked (black) and spiked (blue) with salinomycin.

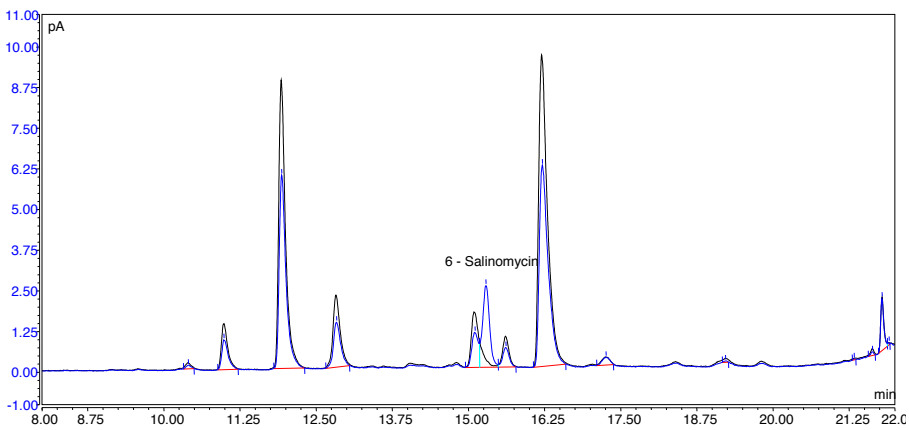
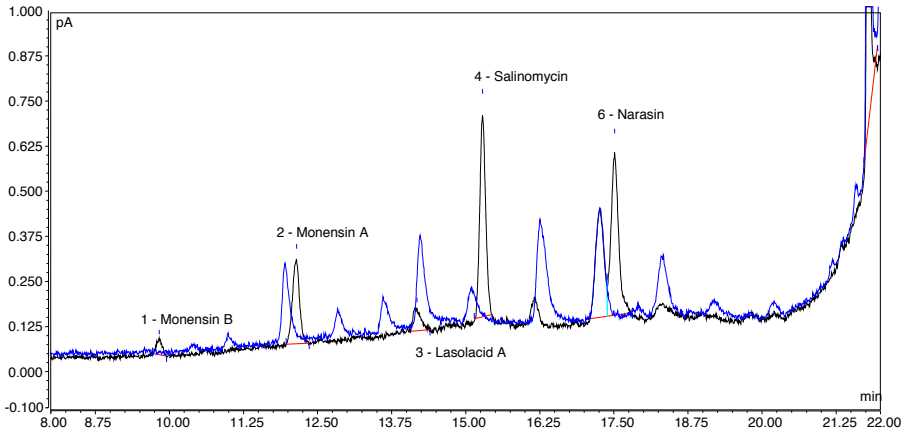


FIGURE 6. HPLC chromatograms of chicken extract SPE eluent (91% methanol), in blue, overlaid with ionophore standard chromatogram (19 ng o.c.), in black.



From the chromatogram overlays, it can be seen that the four ionophoric antibiotics are partially resolved from background peaks. Monensin and salinomycin are partially resolved from two matrix peaks, narasin is partially resolved from a baseline peak, but lasolacid is not resolved under these conditions. For the three major analytes (excluding lasolacid), there is sufficient resolution for quantitation. Modification of the chromatographic conditions (column length/plates, temperature) may further improve the resolution needed for accurate quantitation of monensin, salinomycin, and narasin in food matrices.

Conclusion

A chromatographic method was developed and detailed that provides a direct analysis of four ionophoric antibiotics.

- The method does not require post-column derivatization.
- All USP system suitability requirements were exceeded.
- Linear correlations were created for the three, major antibiotics monensin, salinomycin, and narasin from LOD to 300 ng o.c.
- This method provides the basis for analytical methods that can be used for the direct measurements in food samples.

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