JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of Multiclass Methods for Drug Residues in Eggs: Silica SPE Cleanup and LC-MS/MS Analysis of Ionophore and Macrolide Residues

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A method was developed that is suitable for screening eggs for a variety of nonpolar residues in a single procedure. Residues are extracted by silica solid-phase extraction (SPE). Analysis is conducted via reverse-phase gradient liquid chromatography, electrospray ionization, and tandem ion trap mass spectrometry. For screening purposes (based on a single precursor−product ion transition) the method can detect ionophore (lasalocid, monensin, salinomycin, narasin) and macrolide (erythromycin, tylosin) residues in egg at ∼1 ng/mL (ppb) and above and novobiocin residues at ∼3 ppb and above. Conditions are described for confirmatory analysis based on multiple ions in the product ion spectrum. The extraction efficiency for ionophores was estimated at 60−85%, depending on drug. Recovery of macrolides and novobiocin was not as good (estimated at 40−55% after a hexane wash of the final extract was included), but the method consistently screened and confirmed these residues at concentrations below the target of 10 ppb. The method was applied to eggs from hens dosed with each drug individually. Lasalocid was found to have the highest probability of detection in eggs based on its high ionization efficiency and higher rate of deposition relative to the other drugs. The method is part of a larger scheme to provide surveillance methods for a wide variety of drug residues in eggs.

KEYWORDS: Residues in eggs; ion trap mass spectrometry; ionophore analysis; macrolide analysis; lasalocid; salinomycin; narasin; monensin; novobiocin; erythromycin; tylosin; liquid chromatography−tandem mass spectrometry

INTRODUCTION

Recent advances in mass spectrometry (MS) have expanded the strategies available to monitor for drug residues in edible products. Historically, MS methods for animal drug residues have been used to follow up screening analyses based on microbial inhibition, antigen recognition, or chemical methods based on simpler technology. We are developing and evaluating an approach that uses MS for the initial stage of residue monitoring. This could increase the number of drugs that can be analyzed in one or a few procedures while increasing the specificity of detection relative to other methods.

Liquid chromatography−tandem mass spectrometry (LC-MS/MS) is an attractive technique for residue surveillance because of its inherent specificity and applicability to many compounds. LC-MS/MS instruments have become more sensitive and more widely available in recent years. As a result, the U.S. Food and Drug Administration Center for Veterinary Medicine (CVM) has investigated the simultaneous analysis of a broad range of drug classes for surveillance purposes. Our first effort in this regard focused on drug residues in eggs.

Target residues in eggs were determined by evaluating the analytical literature, data on drug usage in poultry, and reports of residue monitoring. Data for 1995−1999 revealed wide use of ionophores (principally salinomycin) and other drugs (principally nicarbazin) in U.S. broiler chickens (*1*). Lasalocid, monensin, narasin, novobiocin, and salinomycin are approved in U.S. broiler chickens but not in U.S. laying hens, whereas erythromycin and tylosin are approved in both (*2*). Surveys have shown that ionophore drug residues occurred frequently at low parts per billion levels in Swedish eggs (*3*), occasionally at levels well above 100 ppb in the United Kingdom, and frequently at low parts per billion levels in the United Kingdom (<1 ppb) (*4*). Macrolide residues have occurred in Canadian eggs (*5*). Research in Northern Ireland showed that cross-contamination at feed mills is a likely cause of residues in feed and, thereby, in eggs (*6*, *7*).

On the basis of reports such as these, the surveillance method for residues in eggs needed to include the polyether ionophore and macrolide drug classes. Polar drug classes can also be deposited in eggs, for example, fluoroquinolones, tetracyclines, and sulfonamides (*8*). LC-MS can be applied to the detection of all these compounds in a single run, suggesting that one grand method might be feasible. In fact, electrospray LC-MS does

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10.1021/jf040185j
Published on Web 10/20/2004

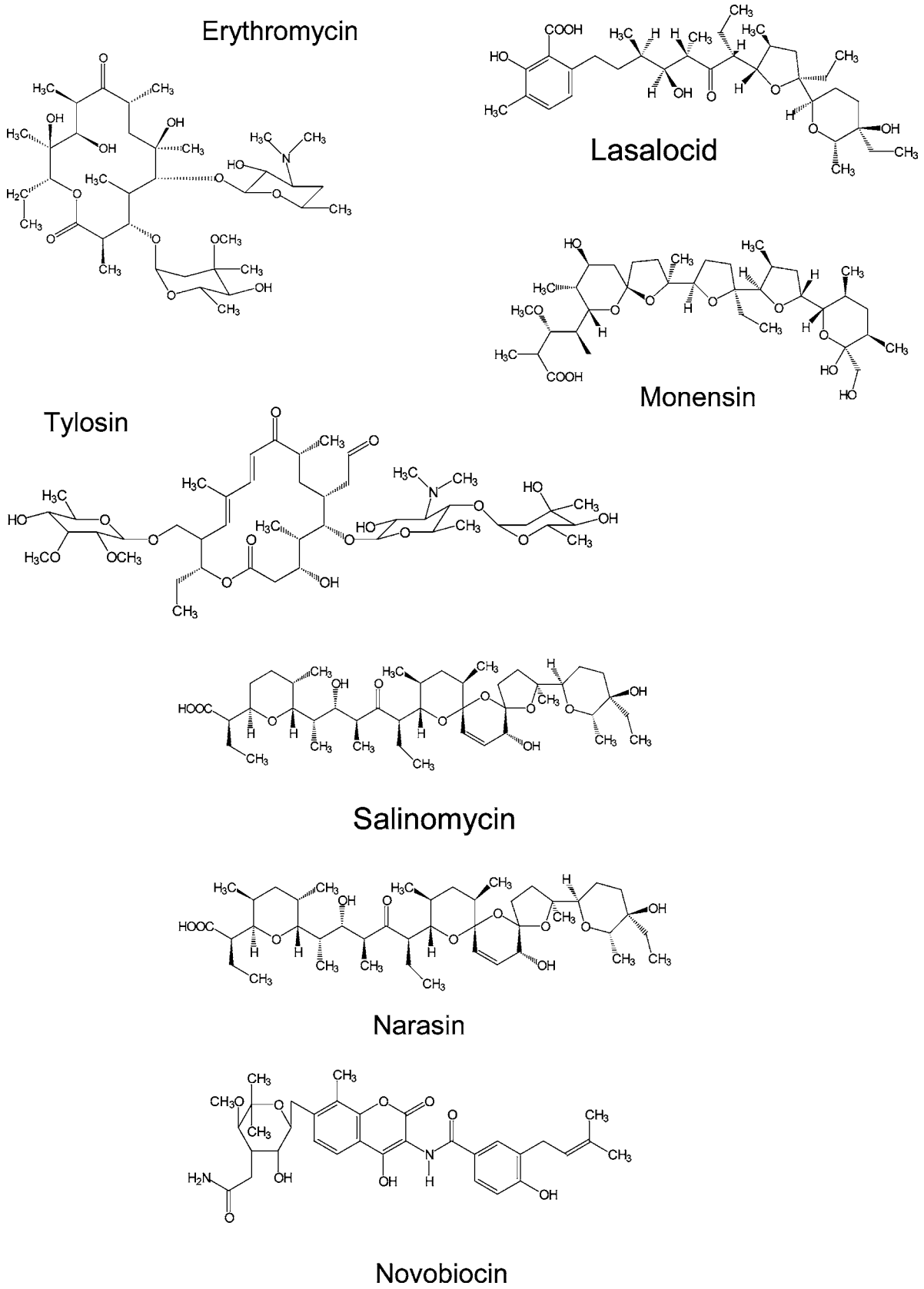

Figure 1. Molecular structures of the seven drugs studied.

ionize standards of all the compounds we tested from six drug classes (the five listed above plus β-lactams) in a single run (data not shown).

Unfortunately, polar and nonpolar drugs differ so greatly that they cannot all be extracted in a single procedure. Therefore, we pursued development of a few methods that can each extract many *related* drugs. The chemical similarities of ionophores, macrolides, and novobiocin suggested that all of these drugs might be amenable to a single procedure (structures are shown in **Figure 1**). For example, a recent publication described an atmospheric pressure chemical ionization (APCI) LC-MS method based on diol-SPE extraction of macrolides and iono-

phores in manure (*9*). On the other hand, we found that polar drugs need to be extracted with a different procedure, the development of which will be reported separately.

It was anticipated that the sensitivity of electrospray ionization (ESI) for ionophores would be more than adequate. The ionophores are so named because of their high affinity for cations, which is key to their physiological activity. The mode of action is to interfere with the exchange of cations across cell membranes. Cation affinity also lends itself to ESI-LC-MS because compounds need to be ionized before they can be detected. The polyether functional groups wrap around the cation, binding it tightly. The ionophore antibiotics indeed yield very strong LC-MS signals and can be detected at very low levels as the $[M + Na]^+$ ion. Macrolide antibiotics can be detected with good sensitivity as the $[M + H]^+$ ion.

The CVM has not set tolerances or concern levels for all drugs in eggs. Further investigation and toxicology studies are needed, and data from our surveillance methods will contribute to such an effort. Ionophores are not approved for use in laying hens, so their presence in eggs might be considered violative at any level. A target concentration of 10 ng/mL (ppb) was selected for method development and validation. Eggs from dosed hens were screened with a preliminary method to select appropriate incurred eggs for validation.

Previous investigators have developed multiresidue LC-MS methods for drugs from single classes, such as ionophores in muscle, liver, and eggs (*10*), macrolides in poultry muscle (*11*), and macrolides in bovine muscle (*12*) and other tissues (*13*, *14*). Silica SPE has been used to extract ionophores (*15*−*17*) and macrolides (*18*, *19*) from food tissues. We applied silica SPE to the extraction of macrolides, ionophores, and novobiocin, another nonpolar drug, in one procedure.

The silica SPE cleanup fits into a larger scheme for detecting drug residues from a wide range of drug classes. This strategy is based on the fractionation of eggs by generic SPE cleanups and the analysis of the extracts by ESI-LC-MS. The LC conditions are based on wide range, generic binary gradients with a reverse-phase LC column. The MS acquisition program is tailored to the variety of drugs recovered by each extraction. This approach was tested previously with a C-18 SPE method for multiple sulfonamides (*21*).

EXPERIMENTAL PROCEDURES

Standards and Reagents. Standards were obtained from the Sigma Chemical Co. as follows: erythromycin (ERY), tylosin (TYL), novobiovin (NOVO), lasalocid (LASA), monensin (MON), salinomycin (SALI), and narasin (NAR). LC grade acetonitrile, hexane, and methanol were obtained from Burdick & Jackson. Formic acid, 88%, was obtained from J. T. Baker. Distilled, deionized water was purified through a Milli-Q system (Millipore) to >18 MΩ resistivity.

Drug standards were dissolved in methanol to a concentration of 1000 μg/mL. Dilutions into methanol were carried out down to 100, 10, or 1 μg/mL as needed for all but lasalocid and monensin. These could be diluted into water at 100 μg/mL and below. Mixtures were prepared at 1 μg/mL for fortification by combining 10 μg/mL solutions and making up to volume in water.

The mobile phase was prepared by diluting 1 mL of formic acid into 1 L of Milli-Q water to yield a 0.1% solution. This was filtered through 0.22 μm nylon filters before use.

Sample Preparation. Fresh whole eggs were homogenized with a Polytron probe for 30 s while immersed in an ice bath. Blended egg samples were stored in polypropylene tubes at < −60 °C. Samples were thawed for extraction at room temperature or in a cold-water bath.

Extraction. Samples were handled with disposable pipets and measured on the basis of volume rather than weight, for convenience and speed. For extraction, 2.5 mL of thawed, blended whole eggs was transferred to a 50 mL polypropylene tube using a 1−5 mL variable pipettor fitted with large bore tips to avoid clogging. Control samples were fortified with appropriate amounts of standard; for example, for fortification at 10 ng/mL (ppb), 25 μL of a 1 μg/mL standard mixure was added. Samples were vortex-mixed for 30 s after fortification.

Acetonitrile (7.5 mL) was added to each tube, and the contents of each tube were vortex-mixed with a multiposition vortexer for 30 min. The tubes were tightly sealed and shaken sideways vigorously for 10 min. The tubes were centrifuged at 5 °C for 10 min at >3000*g*. The supernate solutions were transferred to clean 15 mL polypropylene tubes. Acetonitrile was evaporated under a nitrogen stream in a water bath at 50−55 °C. An additional 7.5 mL of acetonitrile was added to the pellets in the first tube, and the vortex-mixing, shaking, and centrifugation were repeated. The supernates were combined and evaporated to dryness.

The dried extract was taken up in 3 mL of hexane with vortexing for 30 s. Samples were centrifuged at 5 °C for 10 min at >3000*g*, to create a pellet from undissolved solids. The silica SPE cartridges [Supelclean LC-SI, 3 mL, catalog no. 505048 (Supelco, Bellefonte, PA)] were fitted with 20 mL reservoirs using adapters and mounted on a vacuum manifold (Supelco). The cartridges were conditioned with 3 mL of hexane. The supernate was loaded on the SPE cartridge under gravity flow. Then hexane was completely drawn from the cartridges by a brief vacuum, but air was not drawn through. Clean 15 mL polypropylene tubes were placed under each cartridge, and analytes were eluted with 5−6 mL of methanol under gravity flow. Once the solvent drained to the surface of the column bed, vacuum was applied for ∼5 s to completely drain the remaining solvent.

Extracts were evaporated to dryness under a nitrogen stream at 50−55 °C in a water bath. The residue was dissolved in 500 μL of methanol with vortexing for 30 s. (To prepare a spiked control extract for estimating concentration, 475 μL of methanol and 25 μL of the 1 μg/mL standard mixture were added to a dry control extract instead.) An additional 500 μL of water was added to all extracts, which were then vortex-mixed for 10 s and centrifuged at 5 °C for 10 min at >1000*g*.

At this point, extracts usually consisted of a clear liquid above a small, solid, white pellet with a thin yellow layer above it. However, occasionally extracts formed cloudy emulsions and an indistinct pellet. If this occurred, 100 μL of hexane was added, and the extracts were vortexed and centrifuged again, to yield the white pellet and yellow layer as above. The upper hexane layer was discarded in this case.

The clear supernate was transferred to a polypropylene syringe barrel fitted with a Whatman PVDF acrodisc filter, 0.2 μm. The extracts were filtered into amber glass autosampler vials and stored at <10 °C until analysis. A comparison standard was prepared by combining 500 μL of water, 475 μL of methanol, and 25 μL of the 1 μg/mL standard mixture (equivalent to 10 ppb in egg).

Liquid Chromatography−Ion Trap Mass Spectrometry. The LC-MS system was an Agilent 1100 liquid chromatograph combined with a Thermo Finnigan LCQ Classic ion trap mass spectrometer. The LC column was a YMC phenyl cartridge column, 4 × 50 mm, 3 μm silica, fitted with a YMC Direct Connect phenyl guard column, 4 × 20 mm, 3 μm silica (Waters). Injection volume was 100 μL. The mobile phase consisted of a binary gradient at a flow rate of 700 μL/min combining acetonitrile and 0.1% aqueous formic acid (**Table 1**). The LC flow was diverted to waste for the first 4 min after injection and again 16 min after injection.

Table 1. LC Mobile Phase Gradient

	min at pump	ACN, %	0.1% formic acid, %
start	0	20	80
hold	1	20	80
ramp	7	65	35
hold	12	65	35
ramp	13	80	20
hold	16	80	20
ramp	19	20	80
equil	23	20	80

Time-scheduled scan events were used for one or two compounds per segment (**Table 2**). If three or more compounds eluted in a narrow

Table 2. Data Acquisition Parameters, Ion Trap LC-MS/MS

start, min	segment	scan cvent	mode	precursor *m*/*z*	isolation width	collision energy	scan range
0	1 (ERY)	1	MS^2	734.5	5.0	26	200–750
0	1 (TYL)	2	MS^2	916.5	2.0	30	250–925
7	2 (NOV)	1	MS^2	613.2	5.0	50	165–625
12	3	1	MS				600–800
12	3	2	MS^2, data-dependent, largest ion from list	613.4, LASA 693.4, MON 773.5, SALI 787.5, NAR	2.0	50	autoset
12	3	3	MS^2, data-dependent second largest	as above	2.0	50	autoset
12	3	4	MS^2, data-dependent, third largest	as above	2.0	50	autoset

Table 3. Product Ions Monitored for Screening and Confirmation Product ions, MS/MS

residue	precursor *m*/*z*	retention time, min	screen	confirm
$[ERY + H]^+$	734.5	5.4	576	522, 558
$[TYL + H]^+$	916.5	5.9	772	407, 598
$[NOV + H]^+$	613.2	8.7	396	189, 218
$[LASA + Na]^+$	613.4	12.6	577	359, 377
$[MON + Na]^+$	693.4	12.3	657	443, 461
$[SALI + Na]^+$	773.5	12.7	531	513, 431
$[NAR + Na]^+$	787.5	14.1	531	513, 545

time range, *data-dependent* scanning was used (*21*). In this mode, a prescan was carried out in scan event 1, and if ions from the list of ionophore masses appeared above a signal threshold, an MS/MS spectrum was automatically acquired from that *m*/*z* value. Three data-dependent scan events enabled up to three compounds to coelute and still be detected. For ionophore detection, the minimum signal required was 1000 counts, and wideband oscillation was on, to enhance fragmentation of the $[M + Na - H_2O]^+$ ions.

The ESI source was tuned for each different time segment. Some tuning characteristics were the same for all segments: positive ion mode; sheath gas, 90 psi; auxiliary gas, 30 psi; capillary temperature, 225 °C; ESI needle voltage, 5.0 kV; maximum injection time, 500 ms; microscans/scas, 1; automatic gain control on; in-source CID off. Other tuning parameters were optimized with the automatic tuning function of the LCQ while 1 ppm solutions were infused into the mobile phase as follows: 50% acetonitrile for ERY and 65% acetonitrile for NOVO and LASA. These three custom tunes were used for segments 1−3, respectively.

System suitability was evaluated by analyzing the comparison standard at the target concentration. If these data met the signal-to-noise criteria, the sequence of unknowns was considered to be valid. The remaining samples were injected in the following order: spiked control extract, blank, control extract, unknowns, spiked control extract, comparison standard. The column was flushed with 90:10 acetonitrile/water at the end of the day.

Data Processing. Reconstructed ion chromatograms (RICs) were created by summing one to three major product ions for each compound (**Table 3**). An averaged spectrum was created over the range above 20% of full height in the RIC. The same time range was used for averaging control extracts or unknown samples where suspect compounds did not appear. The averaged spectra were used to compare unknown samples and comparison standard.

The signal-to-noise levels for designated product ions per compound (**Table 3**) were evaluated. [The structures of product ions produced by collision-induced dissociation of ionophores have been described (*22*).] Peaks used for screening and/or confirmation had to appear with at least a 5:1 signal-to-noise ratio, measured peak-to-peak without smoothing. A semiquantitative evaluation was carried out by using the control extracts for calibration, with a linear standard curve forced through zero. Percent recovery was based on the response ratio of 10 ppb fortified samples versus controls spiked to 10 ppb after extraction.

Table 4. Estimates of Recovery for Each Drug (Method Performed without Final 100 μL Hexane Back-extraction)

	estimated recovery at 10 ppb fortification ($n = 8$)						
	ERY	TYL	NOVO	LASA	MON	SALI	NAR
% recovery	15	40	50	60	85	80	80
RSD, %	45	20	40	20	20	20	20

Screening criteria were met if the *screening ion* appeared at the correct retention time ±5%, with acceptable signal-to-noise ratio (>5:1) and a semiquantitative result >0.5 ppb. Confirmatory criteria were applied only if the screening criteria were met first. The confirmatory ions had to appear at the correct retention time with acceptable signal-to-noise ratio. The full mass spectrum had to correspond closely to the standard mass spectra acquired the same day. The designated confirmation ions (**Table 3**) had to predominate, above background signals at other *m*/*z* values (*23*).

RESULTS

The method as described above was validated by replicate analysis of control, fortified, and incurred eggs. **Figures 2** and **3** show the selected ion chromatograms and averaged mass spectra for the seven compounds as standards or extracted from 10 ppb fortified control eggs, respectively.

Although the method was not developed for quantitative purposes, this qualitative validation enabled us to estimate extraction efficiency. Recovery was estimated by comparing 10 ppb fortified eggs with control eggs spiked to 10 ppb after extraction (**Table 4**). These values are reported for information only, because this approach is not acceptable for accurate quantitation. Drawbacks to this approach include the lack of a multipoint standard curve and less than ideal recovery for erythromycin, tylosin, and novobiocin. The high relative standard deviations (RSDs) for some drugs resulted from variable ion suppression due to coextractants, as well as the inherent variability of ion trap tandem MS (versus a linear triple-quadrupole instrument).

The data for erythromycin recovery in **Table 4** were acquired prior to the final 100 μL hexane wash of the procedure; this step was later found to improve erythromycin recovery to ∼40% and that of tylosin to ∼50%. In either case, a very high ionization efficiency was observed for erythromycin, and this made up for low recovery. In fact, data shown below demonstrate that ERY performance was sufficiently consistent to compare well with the other drugs, even given low recovery.

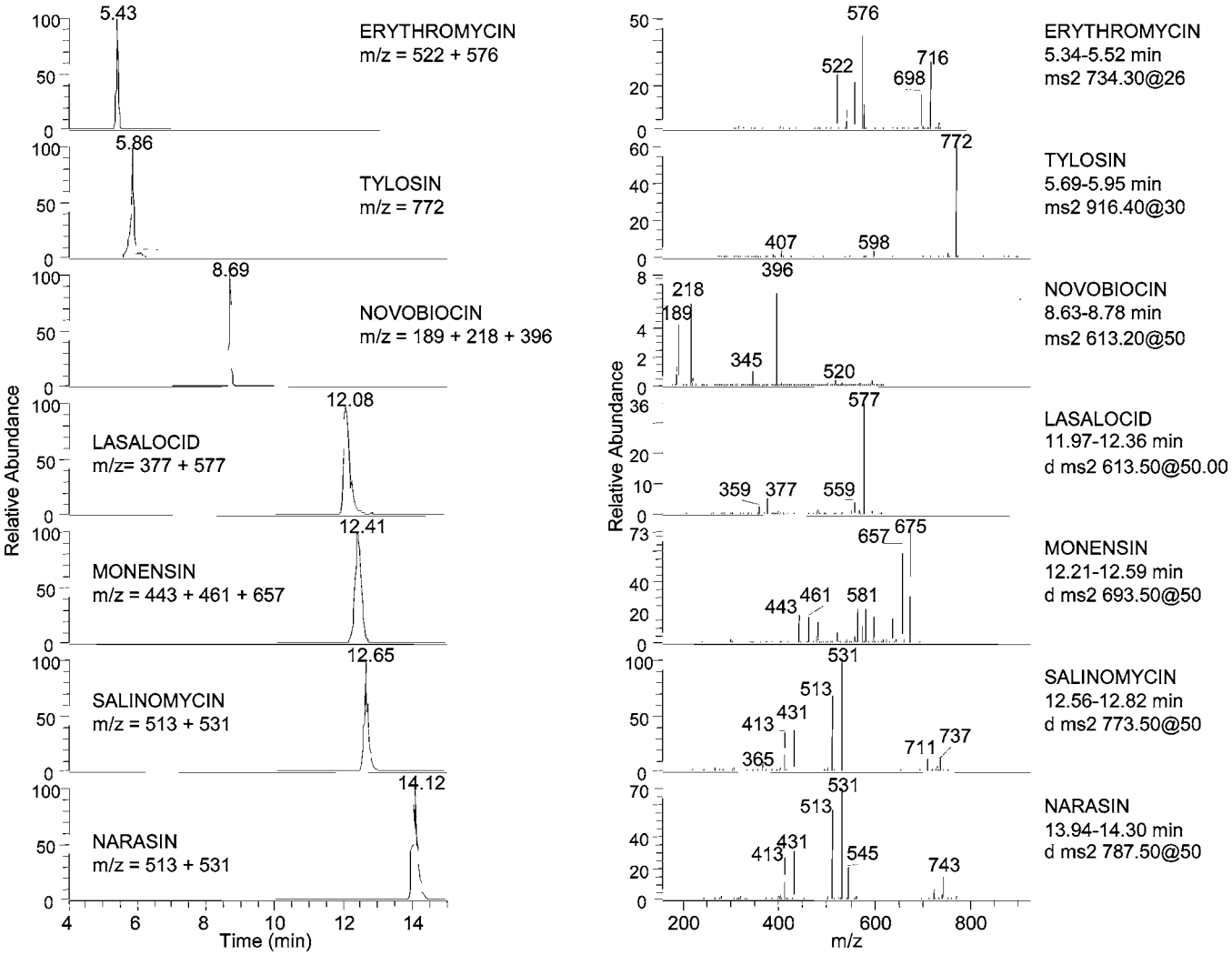

Figure 2. Selected ion chromatograms and averaged mass spectra for comparison standards.

Ideally, full validation requires analysis of tissues containing residues incurred when the animal was dosed with the parent drug. Thus, a dosing study was conducted first to identify incurred eggs for use in method validation. Hens were dosed orally with 20 mg of each drug over 24 h (two 10 mg capsules, 1 day apart). Residue concentration in eggs was monitored for 14 days to select eggs for validation purposes (>10 ppb, if possible). One egg per drug was selected from these sample sets for the validation.

Although the same amount of drug was administered to each hen, widely different levels were observed among the selected eggs during the validation. Drug concentration was estimated by comparing responses from selected samples versus the 10 ppb fortified egg analyzed with each set. Results are shown in **Figure 4**. Error bars represent one standard deviation about the mean ($n = 5$). The number of days after the first dose is indicated on this figure for each drug.

Tables 5 and **6** show the summary results when either the screening or confirmatory criteria were applied, respectively. Performance requirements for confirmation were stricter, and the difference in the results is most evident for ERY, TYL, and NOVO. The overall performance for confirmatory purposes is shown in **Table 7**. Basically, the limit of confirmation depends on the signal-to-noise for the weakest of the confirmation ions. For ERY and TYL, the third ion was quite weak. Of the seven drugs studied, overall response was weakest for NOVO. On the other hand, signal strength from the ionophores was sufficient to enable confirmation well below 10 ppb.

Table 5. Validation Data for Screening Purposes

		no. of samples that met screening criteria						
N	sample type	ERY	TYL	NOVO	LASA	MON	SALI	NAR
5	control	0	0	0	0	0	0	0
8	10 ppb fortified	8	8	7	8	8	8	8
5	incurred	5	5	3	5	5	5	5

Table 6. Validation Data for Confirmatory Purposes

		no. of samples that met confirmation criteria (same sample sets as in **Table 5**)						
N	sample type	ERY	TYL	NOVO	LASA	MON	SALI	NAR
5	control	0	0	0	0	0	0	0
8	10 ppb fortified	8	8	7	8	8	8	8
5	incurred	2	0	3	5	5	5	5

The method was applied to 30 survey eggs in a pilot study. Lasalocid was confirmed in 4 of the 30 survey eggs. **Figure 5** shows the confirmatory data for a survey egg estimated to contain 15 ppb of lasalocid (the highest level found in this set). Tylosin was screened but not confirmed in two survey eggs. **Figure 6** shows the confirmatory data for one of these eggs. The single strong product ion at *m*/*z* 772 enables the screening

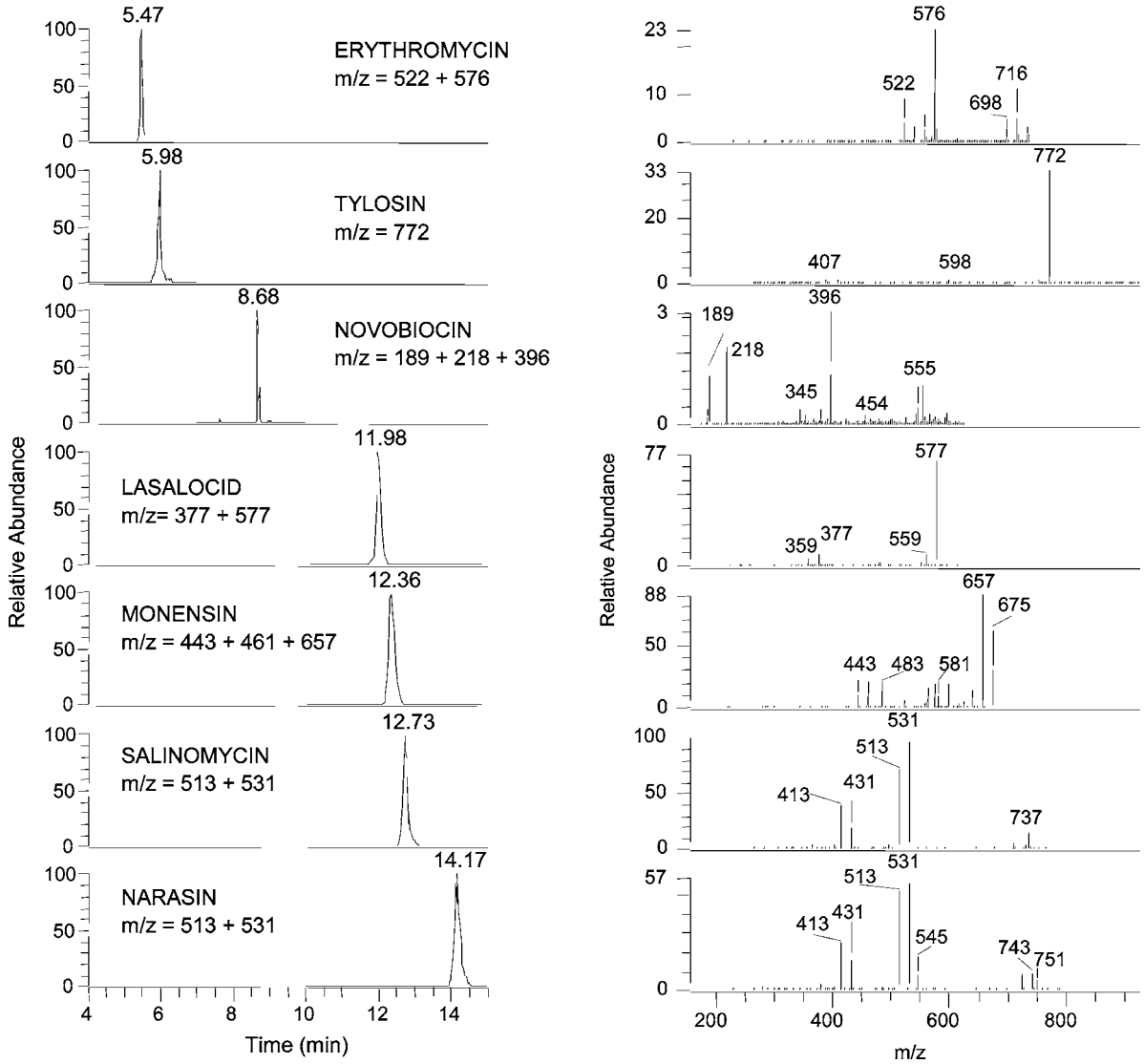

Figure 3. Selected ion chromatograms and averaged mass spectra for 10 ppb fortified control egg.

Table 7. Lower Limits of Performance Evaluated with Fortified Eggs

	lowest fortified level to meet all criteria, in ppb (fortified at 1, 3, and 10 ppb)						
mode	ERY	TYL	NOVO	LASA	MON	SALI	NAR
screening	1	1	3	1	1	1	1
confirmation	3	3	3	1	1	1	1

criteria to be met easily, but the confirmatory ions are quite weak in this compound. This difference in abundance led to significant differences in the lower limit of performance for ERY and TYL (**Table 7**).

DISCUSSION

A variety of method parameters were investigated prior to validation. The instrumental method was optimized by checking each compound's acquisition parameters individually. The technique of ion trap LC-MS/MS is powerful for qualitative analysis, but it has some special characteristics. The "isolation width" parameter defines the mass window that is stored for collision-induced dissocation (CID) to produce MS^n product ion spectra. Wider MS^1 isolation widths (5 amu) were required for erythromycin and novobiocin than for tylosin and the ionophores (2 amu). Sensitivity for ERY dropped 100-fold if the isolation width was too narrow.

We investigated two different schemes for handling the many analytes that could elute in a given time range. *Time-scheduled* scan events work better if only a few residues elute in a given time range. *Data-dependent* scanning is more efficient if many drugs elute in a given time range, because scan events are triggered only if a target ion appears above a threshold in the proper time range.

Method performance was optimized with a variety of experiments before validation. A second acetonitrile extraction of the pellet increased recovery of ionophores. Several types of silica SPE cartridges were tested. The Supelco LC-SI cartridge was selected for the widest range and best recovery of the various drugs tested. Improved SPE performance may correlate with pore size, with 60 Å being somewhat better than 125 Å. The Supelco silica SPE gave clearer extracts, less matrix suppression, and slower flow-through. Methanol was found to be a better SPE elution solvent than ethyl acetate for the full range of these target analytes. We found that the extraction is probably applicable to other macrolide drugs as well; ivermectin was recovered successfully (although it is not a priority for egg surveillance).

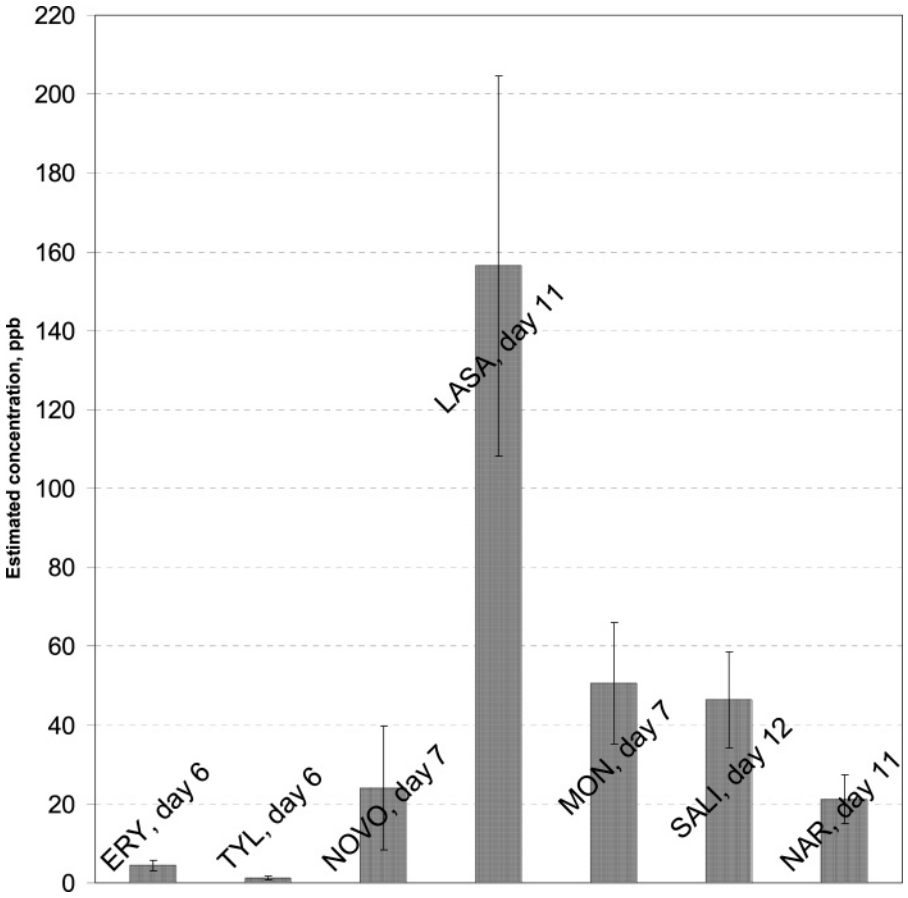

Figure 4. Comparison of residue levels in selected incurred eggs. Individual hens received 20 mg of each drug. Labels show the number of days postdose the egg was collected. Each egg was analyzed five times (error bars show one standard deviation).

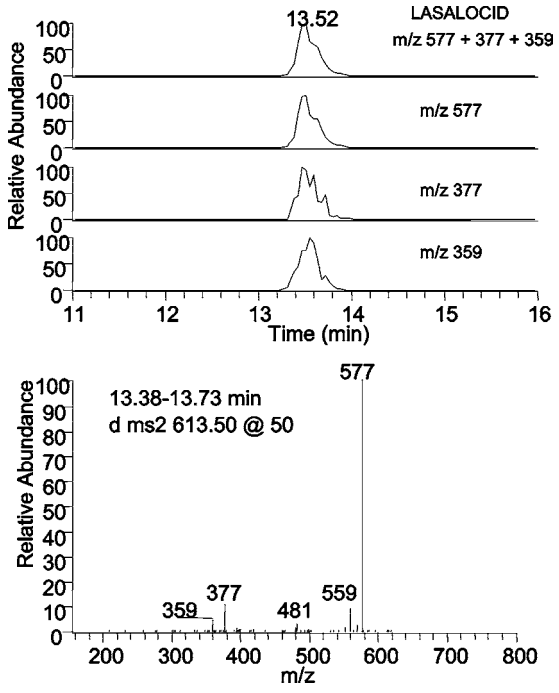

Figure 5. Confirmation of lasalocid in a survey egg. Concentration was estimated at 15 ppb by comparison to a 10 ppb fortified control egg.

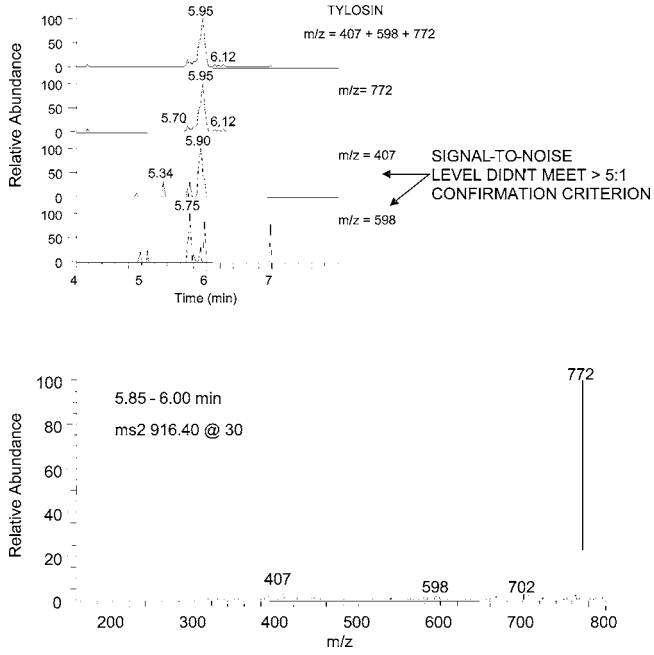

Figure 6. Screen hit for tylosin in a survey egg. Concentration was estimated at 1 ppb by comparison to a 10 ppb fortified control egg. The sample failed to confirm due to low response from the confirmation ions.

Scrupulous efforts to eliminate detergent contamination were required. On one occasion, ionophore LC-MS response was severely suppressed by detergent contamination. The source of this detergent contamination was traced to the reuse of polypropylene SPE reservoirs that had not been completely rinsed.

Mechanical vortexing was substituted for homogenization with a probe, thereby eliminating potential cross-contamination by the probe and increasing throughput. The response from several residue-incurred eggs was found to be quite similar with either technique.

Figure 4 highlights the great differences in the rates of deposition for each drug. When the number of days postdose the egg was laid was taken into account, the relative deposition was roughly LASA ≫ SALI > NARA, MON > NOVO > ERY > TYL. In other words, one might predict that lasalocid is the most likely of these drugs to be found, due to the combination of higher deposition in eggs, good recovery, and good ESI-LC-MS sensitivity.

This extraction method is reliable and has been shown to be applicable as a surveillance tool. Raw data can be quickly scanned to identify possible screening hits, either by checking preset layouts of qualitative data for LC peaks in the correct time window or by quantitative processing to check summary tables for higher measured values.

Control eggs were from hens raised at CVM or from a retail store. Retail eggs were tested for the absence of interfering signals prior to use as controls. However, several eggs from hens raised at CVM in 2000 were confirmed for the presence of lasalocid at roughly 2 ppb. The source of lasalocid in these eggs was not identified.

Early work suggested that novobiocin could be recovered without modifying the method for macrolides and ionophores. However, two new lots of SPE cartridges gave no signals from novobiocin-incurred eggs. Troubleshooting focused on elevated fat recovery from the new silica, which caused an emulsion in the final step. Novobiocin recovery was restored by back-wash of the final methanol/water phase with 100 μL of hexane (the hexane was aspirated off). Furthermore, this step significantly improved the recovery of erythromycin, to roughly 40%. For this reason the hexane wash should be included as a necessary part of the method.

The salinomycin-incurred egg was found to contain methylated salinomycin metabolites, which were detected with the narasin data-dependent scan event (same molecular weight). These could be differentiated from narasin by retention time and mass spectrum. Other than this fortuitous identification, metabolites were not investigated further.

The final procedure requires a full day to extract a set of 20 samples and standards, with LC-MS analysis conducted overnight. The method emphasizes recovery of many drugs along with MS/MS data suitable for confirmation. The second acetonitrile extraction might be skipped to increase throughput, at the cost of lower recovery. The method will be used for further surveillance. It is hoped that application of the method using a more sensitive ion trap or a linear triple-quadrupole will improve quantitative performance. It is expected that this method can be expanded to include other ionophores (e.g., maduramycin) and macrolides (e.g., oleandomycin) with approval for use in broiler chickens but not laying hens (*2*). There is additional work underway to expand this procedure to include other nonpolar drugs, for example, nicarbazin and dimetridazole. Previous work showed that nicarbazin can be recovered by liquid/liquid cleanup directly from the acetonitrile supernate after protein precipitation (*24*) or using the silica SPE cartridge with acetonitrile elution (*13*).

ACKNOWLEDGMENT

We thank Dr. Richard Cullison for dosing the hens and collecting the eggs.

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Received for review April 15, 2004. Accepted August 30, 2004.

JF040185J