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Development of Multiclass Methods for Drug Residues in Eggs: Hydrophilic Solid-Phase Extraction Cleanup and Liquid Chromatography/Tandem Mass Spectrometry Analysis of Tetracycline, Fluoroquinolone, Sulfonamide, and β-Lactam Residues

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A method was developed for detection of a variety of polar drug residues in eggs via liquid chromatography/tandem mass spectrometry (LC/MS/MS) with electrospray ionization (ESI). A total of twenty-nine target analytes from four drug classes—sulfonamides, tetracyclines, fluoroquinolones, and β-lactams—were extracted from eggs using a hydrophilic−lipophilic balance polymer solid-phase extraction (SPE) cartridge. The extraction technique was developed for use at a target concentration of 100 ng/mL (ppb), and it was applied to eggs containing incurred residues from dosed laying hens. The ESI source was tuned using a single, generic set of tuning parameters, and analytes were separated with a phenyl-bonded silica cartridge column using an LC gradient. In a related study, residues of β-lactam drugs were not found by LC/MS/MS in eggs from hens dosed orally with β-lactam drugs. LC/MS/MS performance was evaluated on two generations of ion trap mass spectrometers, and key operational parameters were identified for each instrument. The ion trap acquisition methods could be set up for screening (a single product ion) or confirmation (multiple product ions). The lower limit of detection for screening purposes was 10−50 ppb (sulfonamides), 10−20 ppb (fluoroquinolones), and 10−50 ppb (tetracyclines), depending on the drug, instrument, and acquisition method. Development of this method demonstrates the feasibility of generic SPE, LC, and MS conditions for multiclass LC/MS residue screening.

KEYWORDS: Residues in eggs; ion trap mass spectrometry; sulfonamide analysis; tetracycline analysis; fluoroquinolone analysis; β-lactam analysis; liquid chromatography/tandem mass spectrometry

INTRODUCTION

This method represents the next phase of a long-term program to develop surveillance methods for a wide variety of drug residues in eggs. The main goal of this program is to combine the detection of many different compounds into the fewest methods necessary. We investigated liquid chromatography/tandem mass spectrometry (LC/MS/MS) to take advantage of its well-established sensitivity and specificity. We found that a great many compounds can be ionized by LC/MS in a single run. However, it was not feasible to extract polar and nonpolar compounds in the same procedure due to wide differences in their chemical properties. The first paper in this series describes a silica solid-phase extraction (SPE) procedure for several classes of nonpolar residues in eggs (*1*). The current method uses the same LC column and mobile phase, so extracts from the two methods might be dovetailed in the same analytical batch.

Our laboratory has previously developed methods for multiple residues in single drug classes. For example, a method for fluoroquinolone residues in eggs was developed which used LC and fluorometric detection to gather information about the use or possible misuse of these drugs (*2*). We previously explored the capability of LC/MS/MS to confirm the presence of multiple residues from sulfonamides in eggs (*3*). The current project aimed to simultaneously extract and detect sulfonamides, fluoroquinolones, tetracyclines, and β-lactams in eggs at a target concentration of 100 ng/mL (ppb). The 100 ppb target level was considered by the FDA Center for Veterinary Medicine (CVM) to be a reasonable surrogate for method development purposes, such that methods which could detect residues in eggs at that level would provide valuable surveillance information for human food safety. For example, the regulatory tolerance for chlortetracycline in eggs is 0.4 ppm (*4*).

For the most part, LC/MS multiresidue methods have focused on single drug classes. There have been a number of multiresidue MS methods published for single drug classes in eggs,

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Table 1. List of All Drugs Studied, Showing MH^+ Precursor Ions, Diagnostic MS^2 Product Ions, and Retention Times for the LCQ Classic Method[a]

compound	acronym	RT, min (LCQ Classic)	MH^+	product ions, *m/z*	wideband activation on
sulfaguanidine	SGD	2.4	215.1	*156 + 173*	
amoxicillin	AMOX	4.1	366.1	*349*	114 + 208 + 234
N-acetylsulfanilamide	*N*-AcSA	6.5	215.1	*198*	108 + 134 + 150
cephapirin	CEPH	6.6	424.1	*292 + 320*	
ampicillin	AMP	7.3	350.1	*160 + 174*	
sulfadiazine	SDZ	7.0	251.1	*156 + 174*	
sulfapyridine	SPD	7.4	250.1	*156 + 184*	
oxytetracycline	OTC	7.8	461.1	*426*[b]	
sulfathiazole	STZ	7.8	256.1	*156* + 92 + 108	
sulfamerazine	SMR	7.8	265.1	*156 + 190*	
ciprofloxacin	CIPRO	8.5	332.1	*288* + 245 + 268	
sulfamethazine	SMZ	8.5	279.1	*156 + 204*	
tetracycline	TC	8.7	445.1	410 + 427	*410*
enrofloxacin	ENRO	9.4	360.1	*316*	
sulfamethoxypyridazine	SMPZ	9.4	281.1	*126 + 156*	
sulfamethiazole	SMTZ	9.8	271.1	*156* + 92 + 108	
isochlortetracycline	isoCTC	9.9	479.1	*462*	*462* + 434 + 416
sulfamonomethoxine	SMONO	10.1	281.1	*156 + 215*	
sarafloxacin	SARA	10.4	386.1	*342* + 299 + 322	
difloxacin	DIFLOX	10.7	400.1	*356*	
chlortetracycline	CTC	10.9	479.1	*444* + 462	*444*
sulfachloropyridazine	SCP	10.8	285.1	*156* + 92 + 108	
doxycycline	DOXY	11.6	445.1	*428*	392 + 410 + 428
sulfamethoxazole	SMXZ	11.7	254.1	*156 + 188*	
sulfisoxazole	SIX	12.4	268.1	*156* + 108 + 113	
sulfadimethoxine	SDM	13.4	311.1	*156 + 245*	
sulfaquinoxaline	SQX	13.7	301.1	*156 + 226*	
penicillin G	PEN G	14.7	335.1	*160 + 176*	
cloxacillin	CLOX	18.1	436.1	*160 + 277*	

[a] Italic ions were summed for ion chromatograms. Other product ions were required for confirmation. Wideband activation may increase or decrease the number of product ions (compound-dependent). If only one product ion is listed, MS^3 is required for confirmation (**Table 3**). [b] MS^3.

including tetracyclines (*5*−*8*) and sulfonamides (*3*, *9*). Kennedy et al. showed that isochlortetracycline (iso-CTC) and epi-iso-CTC are metabolites of CTC, and these compounds should be included in residue monitoring in eggs (*10*). Multiple fluoroquinolone residues have been analyzed by LC/MS in catfish (*11*) and chicken tissue (*12*). There have been many reports of multiple β-lactam residues in milk by LC/MS (*13*−*18*).

In one exploration of multiclass methods, cation exchange SPE was used to extract multiple basic residues from eggs (*19*). Other approaches to multiclass extractions involved more complexity. For example, SPE was combined with multiple liquid/liquid extractions to recover 17 compounds from eggs (*20*). Pressurized liquid extraction and a two-step SPE procedure were used to extract tetracyclines and sulfonamides from soils (*21*).

Use of a hydrophilic−lipophilic balance (HLB) polymer solid phase (Waters Oasis HLB) simplifies the extraction of a range of polar compounds from eggs. This SPE phase has been used in other procedures developed for tetracyclines (*22*) or sulfonamides (*23*). Multiclass screening methods for antibiotics in groundwater also employed this material for generic extractions (*24*−*28*).

Our method takes advantage of the flexibility of ion trap MS/MS for qualitative analysis. Either single or multiple product ions may be generated, depending on the diagnostic needs of the analysis. Initial phases of method development were carried out on an early-generation ion trap instrument which is now widely available (Thermo Finnigan LCQ Classic). We recently used a later generation of the same instrument (Thermo Finnigan LCQ Deca XP Plus) for several experiments reported here.

A recent paper by Kan and Petz reviewed what is currently known on the deposition of orally dosed drugs and metabolites in eggs and their distribution between yolk and egg white (*29*). They concluded that it may not be possible to predict (on the basis of in vitro data) whether or how much of an orally dosed drug would be deposited in egg white or yolk. Consequently, our work required the generation of residue-incurred eggs to determine what form such drug residues might take before method validation is undertaken.

We did not find reports of MS-based analyses that β-lactam residues occur in eggs following oral administration. In contrast, microbiological methods showed residues following intramuscular injection (*29*, *30*) and trace levels after oral administration in water (*31*). Therefore, it was necessary to investigate whether β-lactam residues could be found in eggs by LC/MS/MS after oral dosing.

EXPERIMENTAL PROCEDURES

Dosing. White leghorn hens (Dekalb Delta) were used in this study. Birds were provided a standard ration and environmentally housed to maintain a normothermic environment and lighting regime. Control eggs were obtained from laying hens prior to dosing. Individual hens were dosed orally with single drugs in capsules at 10 mg/day for 2 days, approximately 24 h apart (one hen per drug). Eggs were collected for about 10 days after the initiation of dosing.

Standards. Standards were obtained from Sigma Chemical Co. (St. Louis, MO). Stock standards were prepared at 1000 μg/mL. Drugs tested are shown in **Table 1**. Stock solutions of tetracyclines and sulfonamides were prepared in methanol, fluoroquinolones in 2% acetic acid in water, and β-lactams in acetonitrile. Intermediate standards were prepared by dilution to 100 and 10 μg/mL in water purified through the Milli-Q system (Millipore, used for all subsequent references to water). Individual working standards and various working mixtures were prepared by dilution of intermediate standards with water to yield solutions of 1.0 μg/mL per drug. Working standards were stored at

<−10 °C. β-Lactam solutions were subdivided and stored at <−60 °C, and each portion was discarded after use to avoid degradation on multiple freeze−thaw cycles.

Reagents. A 0.1 M sodium succinate solution was prepared by dissolving 11.8 g of succinic acid (Sigma) in 1 L of water. (Sodium succinate is one of several diacid salts, including sodium ethylenediaminetetraacetic acid, which chelate metal ions that otherwise would interfere with extraction of tetracyclines.) The pH was adjusted to 3.5 by addition of 10 N sodium hydroxide. The 0.1% formic acid mobile phase was prepared by diluting 1 mL of formic acid (88%) to 1 L with water and filtering through a 0.22 μm nylon filter.

Sample Preparation. Whole eggs (yolk and albumen combined) were blended with a Polytron homogenizer (Brinkmann) while immersed in an ice bath. Blended samples were extracted immediately or stored at <−60 °C.

Extraction. A 2 mL volume (approximately 2 g) of blended whole egg was transferred to a 50 mL polypropylene tube. Fortified samples were prepared by adding the appropriate volume of 1 μg/mL working standard or mixture (for example, 200 μL = 0.1 μg/g, or ppm). After thorough vortex mixing, 18 mL of sodium succinate buffer was added, and the mixture was homogenized with a Polytron for 30 s. The sample was centrifuged for 10 min at > 3000*g*, which resulted in a cloudy solution and a small pellet.

A 20 mL reservoir with a polypropylene frit was mounted above an Oasis HLB SPE cartridge, 3 cm^3, 60 mg of sorbent (Waters) using an SPE cartridge adapter. The SPE cartridge was conditioned with 3 mL of methanol followed by 3 mL of water. Half of the supernatant (10 mL) was transferred to the sample reservoir, being careful to avoid disturbing the pellet or transferring any floating fat. The reservoir frit prevents clogging of the SPE cartridge. The liquid was drawn through the SPE cartridge under vacuum using a moderate flow rate of 2−3 drops/s. The sample reservoir and adapter were removed, and the cartridge was rinsed with 1 mL of water. The cartridge and tubing were completely drained of water by briefly applying a vacuum, but air was not drawn through it for an extended period of time. All washes were discarded.

Analytes were eluted with 3 mL of methanol into a fresh 15 mL graduated polypropylene centrifuge tube. A vacuum was applied, if necessary, to start methanol flow through the SPE cartridge. About 1 mL of water was added to act as a keeper during evaporation. Methanol was evaporated from the extract using nitrogen in a water bath at 40−45 °C until the volume was reduced to 0.3−0.5 mL. It was critical to not let the extract go to dryness. Water was added to bring the final volume to 1 mL. The extract was vortexed and then centrifuged at >2500*g* for 10 min. The supernatant was carefully poured into a disposable syringe barrel fitted with a PVDF Acrodisc filter, 0.2 μm (Whatman). The tube was tilted so the precipitate remained on the upper side during pouring. The extract was filtered into amber glass autosampler vials. If analysis could not be carried out within 24 h, the extracts were stored at <−60 °C.

A comparison standard (no matrix) was prepared by combining half the fortification volume of the working standard with sufficient water in an autosampler vial to yield a final volume of 1 mL. For example, 900 μL of water was combined with 100 μL of 100 μg/mL working standard for a solution equivalent to 100 ppb in egg. The half-volume adjustment was necessary for equivalence, since only half the extract was processed by SPE. Also, extra control extracts were fortified after extraction with half the fortification volume of the working standard (standards in matrix). Comparison of the response in controls fortified pre- and postextraction gave an estimate of absolute recovery, while comparing standards in matrix to a comparison standard gave an estimate of the matrix effect on LC/MS ionization.

Liquid Chromatography/Tandem Mass Spectrometry. The LC column was in both cases a phenyl cartridge column, 4 × 50 mm, 3 μm silica, fitted with a guard column of the same phase (YMC). For the LCQ Classic, a binary gradient was carried out at a flow rate of 700 μL/min. The initial conditions were 97:3 0.1% formic acid/acetonitrile. The mixture was ramped linearly to 60:40 at 15 min, to 35:65 at 18 min, and to 85:15 at 21 min, held for 2 min, and then ramped over 1 min to 97:3 for at least 5 min of equilibration before the next injection. The divert valve was programmed to send the LC flow to waste for the first 2 min after injection and again after all the analytes of interest had eluted (20 min). The injection volume was typically 75 μL, but this was adjusted between 50 and 100 μL depending on the expected concentration. For the LCQ Deca XP Plus a similar binary gradient was carried out at a reduced flow rate (500 μL/min) to prevent condensation in the spray chamber. The 0.1% formic acid/acetonitrile mixture was ramped linearly from 97:3 at 0 min to 60:40 at 11 min, to 35:65 at 13 min, and to 15:85 at 14 min, held for 2 min, and then ramped over 2 min to 97:3 for at least 5 min of equilibration before the next injection. The divert valve was programmed as before. The injection volume was 60 μL.

LC/MS Tuning. Positive ion electrospray ionization was used in all cases. Each acquisition method used only a single tune file for the entire period that analytes eluted. For the LCQ Classic, the sheath gas setting was 90 and the auxiliary gas setting was 30 (arbitrary units). The capillary temperature was 225 °C, and the ESI needle voltage was 5.4 kV. The maximum isolation time was 500 ms. Acquisition methods called for one microscan per scan saved to disk. After these settings were fixed, a 10 μg/mL solution of OTC was infused into LC effluent while 80:20 0.1% formic acid/acetonitrile was pumped at 700 μL/min. All other source parameters were optimized using the XCalibur automatic tuning procedure while the OTC product ion transition *m*/*z* 461 → *m*/*z* 443 was monitored. This tune file provided acceptable ionization conditions for the other drugs in the method. The LCQ Deca XP Plus tuning parameters were set as follows: sheath gas, 75; auxiliary gas, 30; ion transfer (heated capillary) temperature, 340 °C, ESI needle voltage, 5 kV; maximum isolation time, 25 ms; one microscan per scan saved to disk. Other tuning parameters were optimized automatically with OTC infusion as for the LCQ Classic described above.

Collision-Induced Dissociation. On the LCQ Classic, the isolation width (the *m*/*z* window selected for CID) was 1.6 amu for all compounds except amphoteric β-lactams (AMOX, AMP, PEN G, CLOX), which required a 5 amu isolation width to avoid a decrease in sensitivity. The collision energy for CID was set at 50 (arbitrary units), and wideband activation was off in all cases.

Acquisition Method. The acquisition method was designed to cover a broad time range, while maximizing sensitivity for each analyte and allowing some flexibility in retention time. The acquisition period was divided into 1.0 min segments. Analytes were allotted to two or three adjacent time segments centered on their average retention time. For example, a compound eluting at 11.2 min was allotted to two time segments using identical parameters (yielding one chromatogram): 10−11 and 11−12 min.

The acquisition strategy within each segment depended on how many target analytes were expected to elute in each 1 min period and how fast the product ion spectra could be acquired. If a small number of compounds were expected, then "time-scheduled" scan events were used as follows: Specific MS^n parameters were predetermined, and the instrument scanned them iteratively. For a larger number of compounds in a segment, data-dependent scanning was used. The exception to this general scheme was OTC, which required a time-scheduled MS^3 scan event to avoid an interference at *m*/*z* 461. OTC was monitored as follows: *m*/*z* 461.1 → *m*/*z* 443, isolation width 1.6, collision energy 23%, followed by *m*/*z* 443 → *m*/*z* 426, isolation width 4, collision energy 28%.

Data-Dependent Scanning. In this mode, product ion MS^2 scans for a given compound were only collected if a predetermined molecular ion produced >1000 counts in an MS^1 prescan. The predicted MH^+ mass for analytes eluting in a given time segment were added to the mass list for that segment. Up to three data-dependent MS/MS scan events were allowed per segment to accommodate up to three coeluting drugs. Using the data-dependent mode enabled more scans per peak with the LCQ Classic. By contrast, the LCQ Deca XP Plus was found to obtain similar quality spectra at a much faster rate. Time-scheduled acquisition could be used with the Deca XP Plus with up to 10 scan events per time segment, whereas the largest number of time-scheduled events used with the LCQ Classic was only three.

Interface Optimization. The best sensitivity with the LCQ Classic was obtained after the API stack was cleaned and the heated capillary was reamed out. For both instruments, the fused silica transfer line

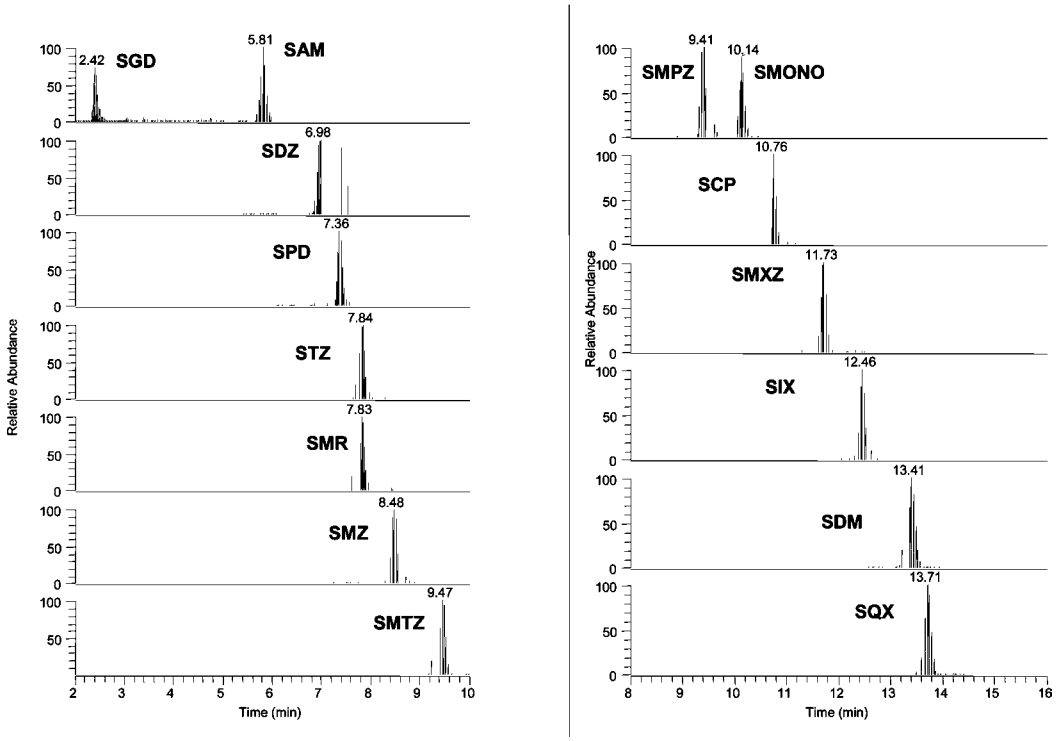

Figure 1. Fifteen sulfonamides extracted from a 50 ppb fortified egg. Ion chromatograms represent the *m*/*z* 156 product ions from the respective MH^+ ions (LCQ Classic).

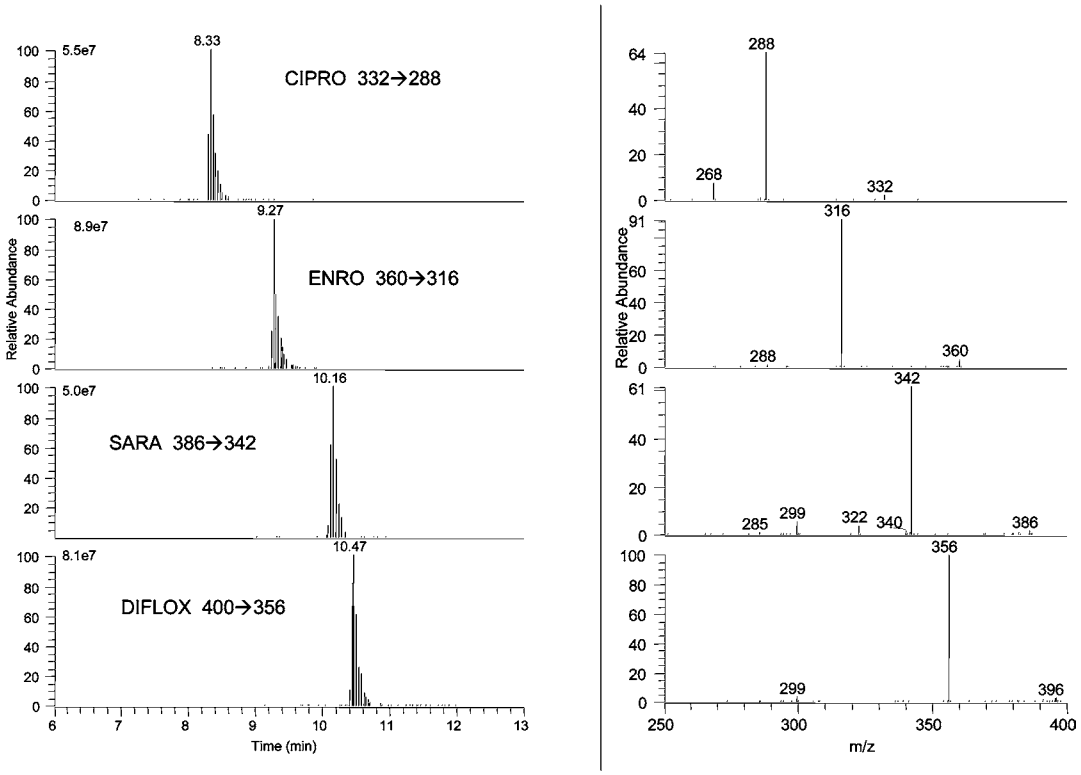

Figure 2. Four fluoroquinolones extracted from a 100 ppb fortified egg: selected product ion chromatograms and mass spectra corresponding to the LC peaks (LCQ Classic).

was set flush with the end of the stainless steel needle after the polyimide coating was burned off with brief exposure to flame.

Injection Sequence. One gradient cycle was performed with a water blank before any injections were made. At least one injection of comparison standard equivalent to 100 ppb in eggs was made to assess the system suitability. On the LCQ Classic, at least one control extract was injected first because this method gave matrix enhancement effects on the ionization of some compounds (tetracyclines, most notably). The preliminary "conditioning" of the system tended to stabilize the matrix enhancement effect throughout the run (see the Results and Discussion). Comparison standards, blanks, fortified samples, and standards in a matrix were interspersed through the sequence to help

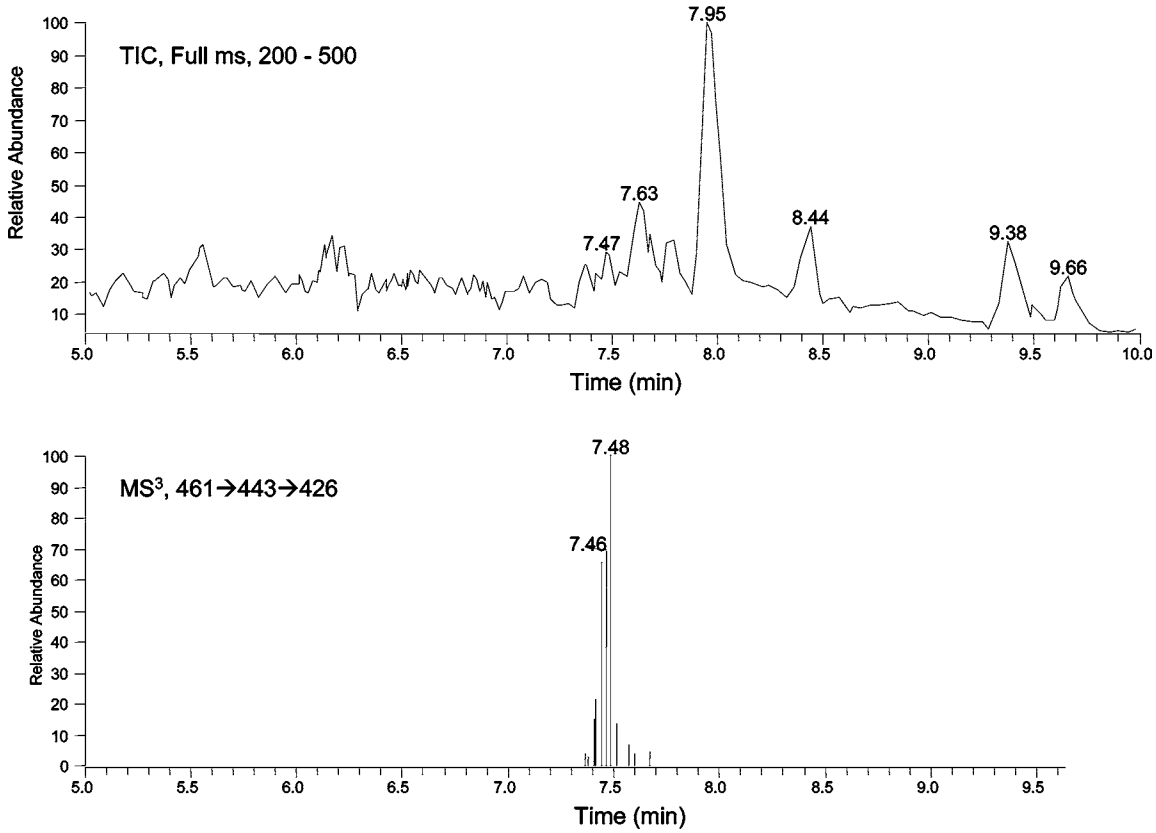

Figure 3. Oxytetracycline incurred egg, estimated 20 ppb: comparison of MS^1 prescan and time-scheduled MS^3 scan events (LCQ Classic).

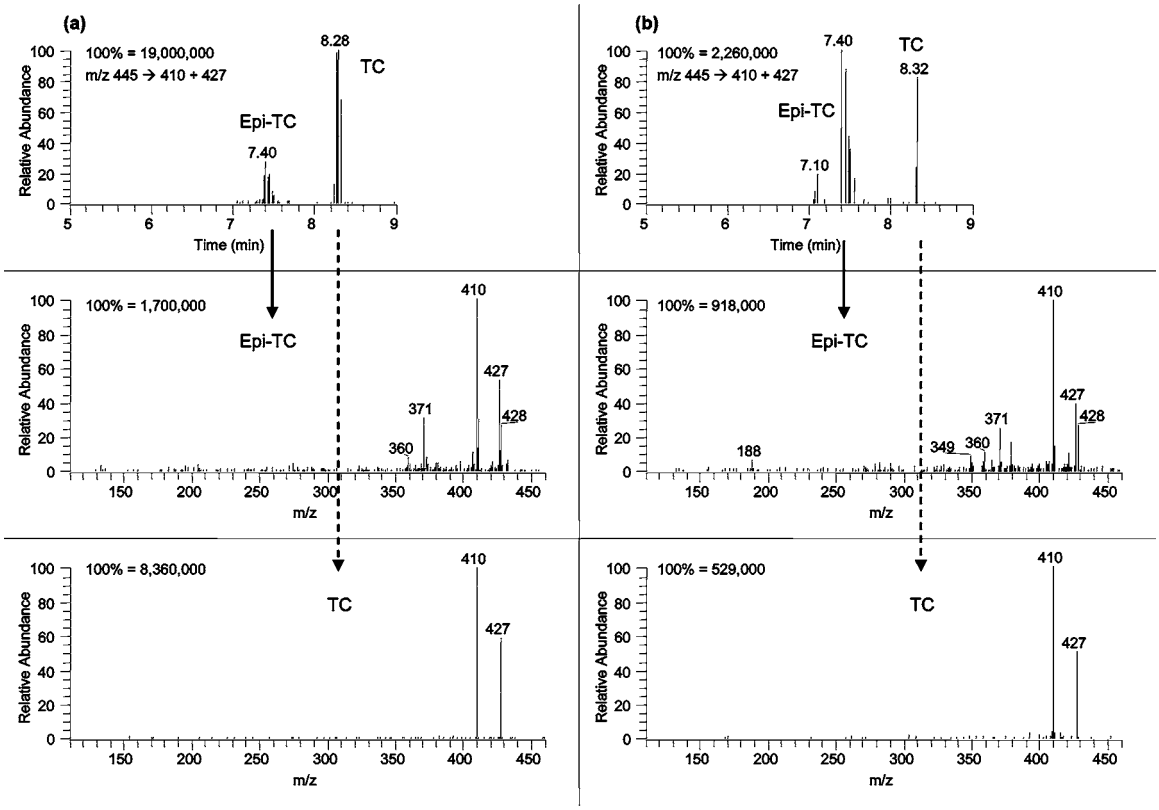

Figure 4. Tetracycline response from two eggs: (a) 100 ppb fortified, (b) incurred egg, from hen dosed orally with tetracycline, estimated at 35 ppb (LCQ Classic).

monitor the instrument performance. At the end of the set, the column and LC lines were flushed with 10:90 acetonitrile/water for at least 10 min.

Data Processing. Data were inspected with the qualitative processing package of the Xcalibur software. Data "layouts" were created that displayed ion chromatograms and mass spectra for each compound

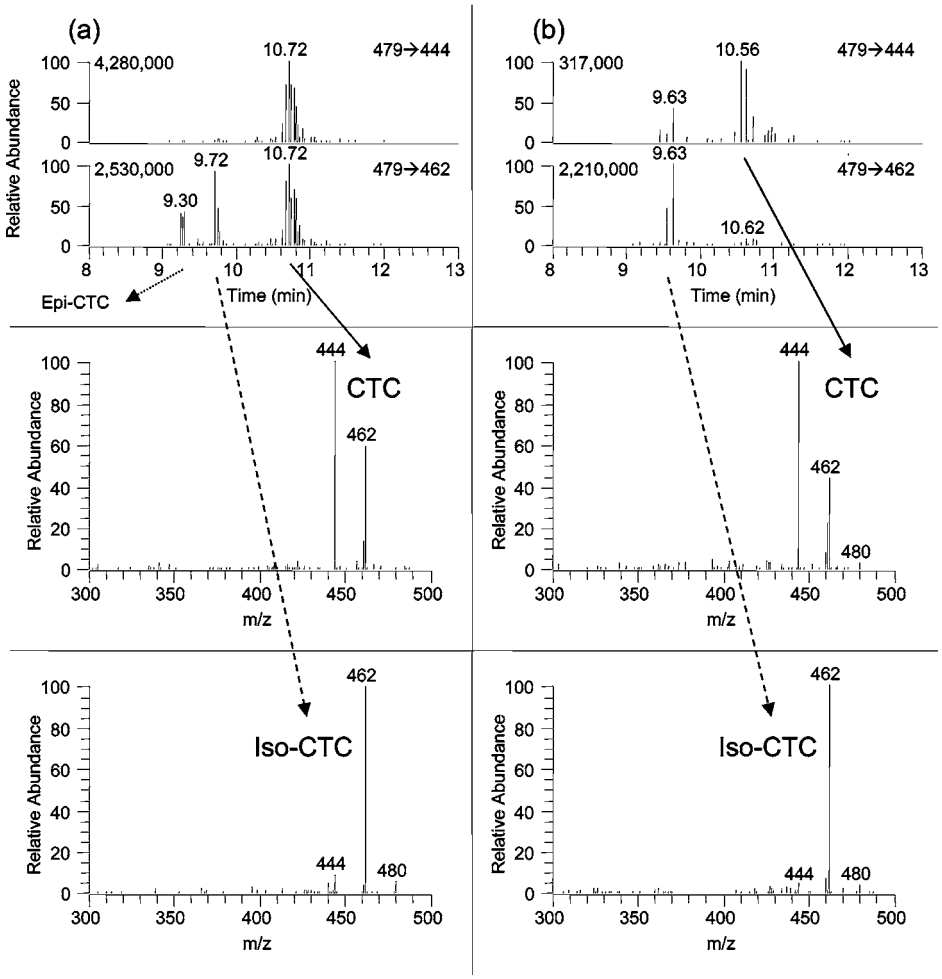

Figure 5. Comparison of chlortetracycline response from 100 ppb fortified (a) and CTC incurred egg, estimated at 25 ppb (b) (LCQ Classic).

across its allotted time range. The ion chromatograms were composed of the summed diagnostic product ions (**Table 1**) shown without smoothing, so the appearance of a peak at an appropriate retention time tentatively indicated the presence of a target drug. Averaged mass spectra were created across these peaks. The data were also processed with the XCalibur quantitation package to estimate concentration and recovery (see the Discussion).

Identification Criteria. Screening was based on the appearance of diagnostic ions (**Table 1**) in consecutive scans at a retention time that matched the contemporaneous standard, ±5%. Peaks from summed diagnostic ions needed to show signal-to-noise (S/N) levels >3 times that of the chemical background. The base peak in the averaged spectrum needed to be one of the diagnostic ions, with S/N > 3 compared to that of the chemical background.

In many cases the full product ion spectra obtained by either data-dependent or time-scheduled acquisition were also sufficient for confirmation according to CVM's guidance document on regulatory confirmation (*32*). That is, their spectra contained multiple structurally specific ions at reproducible abundances. Chromatograms of each diagnostic product ion (**Table 1**) needed to show S/N > 3, and relative abundances needed to correspond to contemporaneous standards. If the product ion spectrum contained only one major ion (albeit an advantage for screening purposes), its fragmentation was enhanced for confirmation in a reinjection using wideband activation, MS^3, or both (see the Discussion).

RESULTS

This method was designed to obtain qualitative surveillance information at or above 100 ppb. Quantitative performance was evaluated only to estimate recovery, lower detection limits, and incurred residue concentrations. Indeed, the CVs for interday analyses with this method ranged widely, from ~10% to >30% ($n \geq 14$ for all drugs). Therefore, the quantitative results are useful only for estimating a concentration range.

Recovery. Extraction efficiency was estimated by analyzing sets of control egg samples with standards added before or after extraction. The chromatograms for diagnostic ions listed in **Table 1** were integrated and quantified using standard in a matrix as a single-point calibrant. This approach was not designed for quantitation of the drug level, although it did compensate for any matrix effects on ESI-LC/MS. The fortification level was 100 ppb in >80% of cases, and nearly all others were at 50 ppb. The standard in matrix concentration was equivalent to 100 ppb in virtually all cases. Recoveries for each drug class ranged as follows: sulfonamides, 70−80% ($n > 20$ in each case), except for SGD, SCP, and SIX (45−65%, $n > 20$); tetracyclines, 45−55% ($n > 15$); fluoroquinolones, 70−80% ($n \geq 14$). β-Lactam recoveries were lower and more variable, with AMOX and CEPH at <25% and AMP, PENG, and CLOX at 30−50% ($n \geq 14$).

Lower Limits of Performance. The chromatograms and mass spectra from eggs fortified at 10−100 ppb were examined according to the identification criteria. The lower limits for screening purposes with the Deca XP Plus were estimated at 10−20 ppb for fluoroquinolones, sulfonamides, and tetracyclines. The screening limits on the LCQ Classic were estimated at 10−20 ppb for fluoroquinolones and sulfonamides and 25−50 ppb for tetracyclines and sulfaguanidine. The β-lactam screening limits were approximately 50 ppb with the LCQ Classic. However, the β-lactam performance with the Deca XP

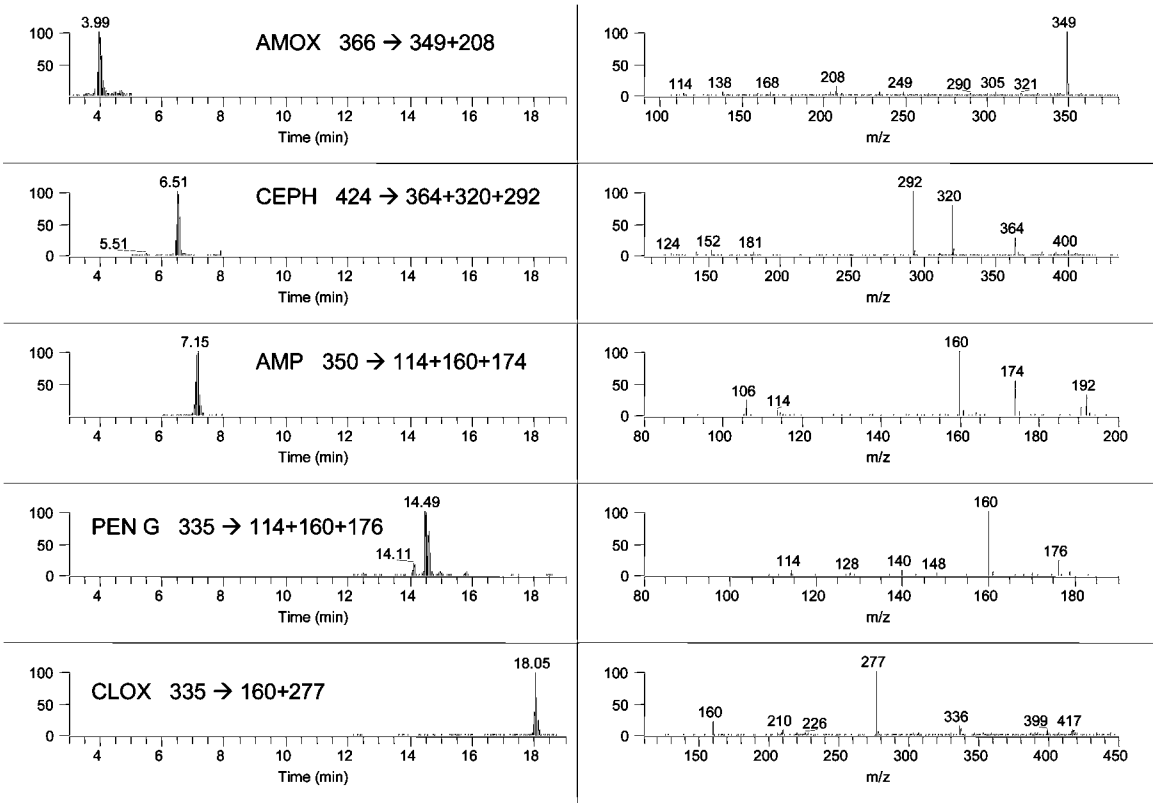

Figure 6. Example of an extract from egg fortified with 100 ppb β-lactams: summed diagnostic ion chromatograms and mass spectra corresponding to the LC peak (LCQ Classic).

Plus was considerably worse, most likely due to the compressed chromatography, which led to more matrix suppression. Concentration limits for confirmation ranged from 2- to 5-fold higher than screening limits, depending on the signal strength and number of additional product ions required for confirmation.

Incurred Residue Concentrations. An estimate of actual concentration in the incurred samples was made by comparing test samples against the 100 ppb fortified control run with each batch. Again, this step was useful for range finding while compensating for matrix effects.

Sulfonamides. **Figure 1** shows composite results from 15 sulfonamides fortified in a control egg at 50 ppb. The ion chromatograms represent only *m*/*z* 156 formed from the respective MH^+ ions. The signals at about 7.3 min in the SDZ trace represent "cross-talk" from the SPD scan event. The two compounds differ in molecular mass by only 1 amu, but the isolation width was 5 amu wide to accommodate β-lactams. The two compounds can be differentiated because their MS/MS spectra are different (**Table 1**) (*3*).

Sulfanilamide and sulfacetamide are heavily metabolized by the hen to *N*-AcSA), which is the appropriate marker residue for their use (*3*, *33*). *N*-AcSA was synthesized, and several experiments were carried out on the LCQ Deca XP Plus to characterize this compound. Recovery through the procedure was found to be about 80%. Under the conditions shown in **Table 1**, the screening ion for *N*-AcSA is *m*/*z* 198 (*3*).

Fluoroquinolones. **Figure 2** shows results from a control egg fortified with four fluoroquinolones at 100 ppb. The diagnostic product ions correspond to loss of CO_2 (44 Da) from MH^+. Ciprofloxacin was detected as a metabolite in eggs from hens dosed with enrofloxacin. Likewise, sarafloxacin was detected in eggs from hens dosed with difloxacin.

Tetracyclines. Although the same dosage levels were administered to hens, the levels of incurred residue were significantly higher for DOXY than for CTC, OTC, or TC. Apparently the less polar compound was deposited to a greater extent. **Figure 3** shows a time-scheduled MS^3 screening chromatogram for oxytetracycline in an incurred egg, analyzed with the LCQ Classic. Comparison to the MS^1 full scan data (upper trace) shows the clear signal-to-noise advantage of this approach. The concentration of OTC was estimated at 20 ppb by comparison to a 100 ppb fortified control egg.

Figure 4 compares results for tetracycline in a 100 ppb fortified egg and an incurred egg obtained with the LCQ Classic. The peaks at 7.4 min are due to epi-TC. Epimerization at C-4 occurs both in the hen and during the extraction, so the epi-TC concentration in the incurred egg appears somewhat higher relative to that of the parent TC. A direct measurement of concentration under this circumstance is difficult. Comparing the sum of the TC and epi-TC responses in fortified and incurred extracts suggests the incurred concentration was approximately 35 ppb, as a combination of TC and epi-TC.

Figure 5 shows similar comparisons for chlortetracycline on the LCQ Classic. In the fortified egg (a) the major peak is due to CTC at 10.72 min. The additional peaks at 9.3 and 9.72 are due to epi-CTC and iso-CTC, respectively. These alternative forms occur because CTC degrades somewhat during sample processing, but also as metabolites in egg. Iso-CTC is the major metabolite of CTC found in eggs (*10*). Formation of iso-CTC occurs when the C-6 hydroxyl group rearranges to become more tightly bound, thus not easily lost in MS/MS. Conversely, the C-6 hydroxyl is readily lost (as H_2O) from the parent CTC, yielding the additional product ion at *m*/*z* 444 (*34*). With wideband activation off, the iso-CTC MS/MS spectrum consists only of one ion at *m*/*z* 462 due to loss of NH_3 (data not shown). In **Figure 5** the incurred concentration was estimated at 25 ppb by assuming the iso-CTC response was comparable to that of CTC in a 100 ppb fortified egg.

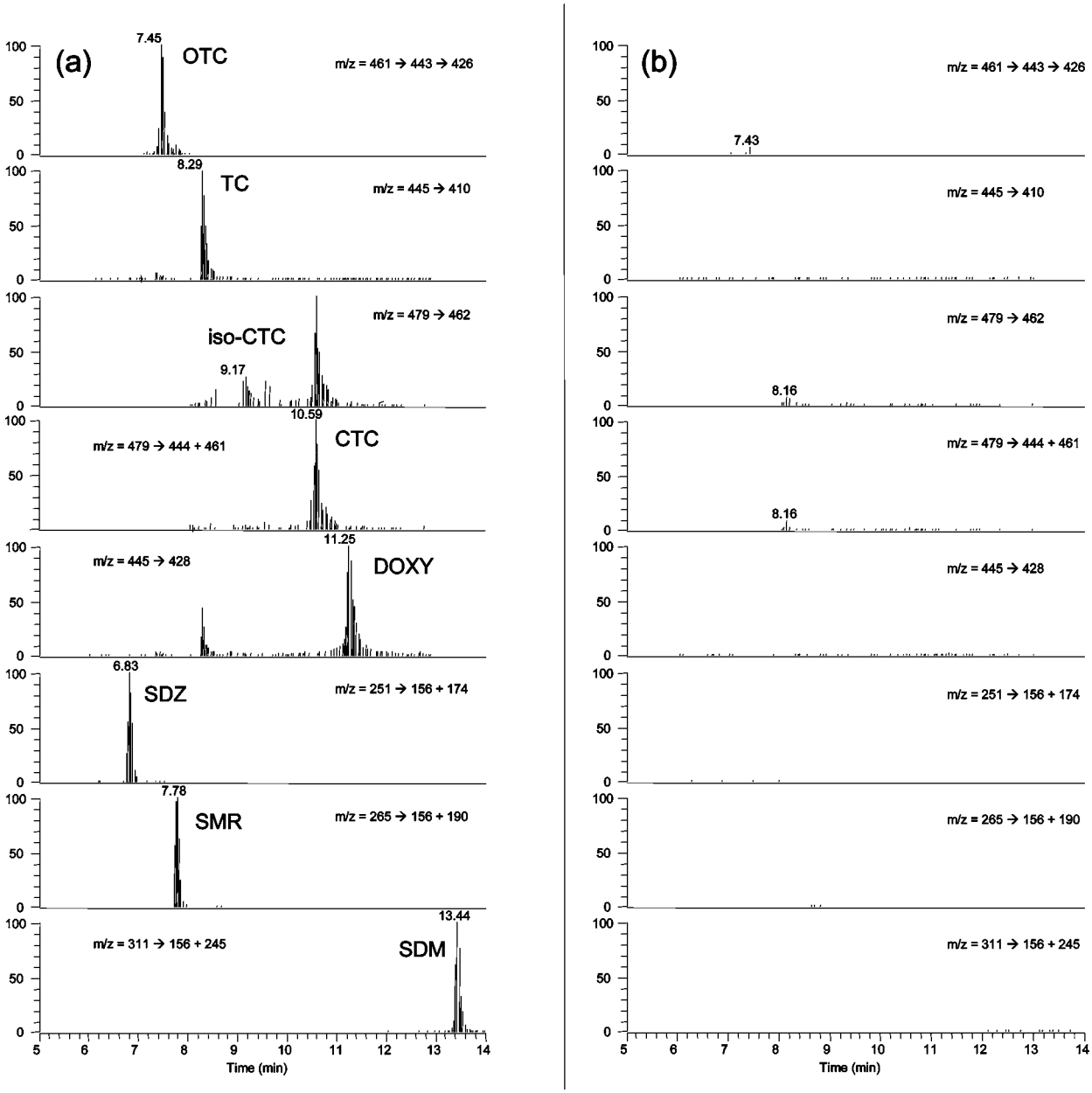

Figure 7. Multiclass screening analysis of drugs fortified in egg at 100 ppb: tetracyclines and selected sulfonamides (a), compared with a control egg extract (b), plotted to the same scale for each drug (LCQ Classic).

β-Lactams. It was found that β-lactams could also be recovered by the Oasis HLB SPE cartridge, so they were included in the detection scheme to demonstrate performance with four classes of drug residues. **Figure 6** shows results obtained with the Oasis SPE extraction and the LCQ Classic for a control egg fortified with a 100 ppb concentration of five β-lactams. Penicillin G and cloxacillin response suffered more from matrix suppression than that of all other drugs in this method.

We investigated whether β-lactam residues were deposited in eggs after oral dosing. This work was carried out with a validated C-18 SPE extraction (*3*) and LC/MS/MS acquisition parameters designed only for AMOX, AMP, CEPH, PEN G, and CLOX (β-lactam validation data not published). Under these conditions, limits of detection were approximately 5−10 ppb. Laying hens were orally dosed with β-lactam antibiotics, and their eggs were analyzed for the presence of β-lactam parent drugs. However, β-lactam residues were not detected with this procedure, casting doubt on whether the β-lactams need to be included in the final surveillance method.

Figures 7 and **8** demonstrate the simultaneous extraction and analysis of multiple drug classes at 100 ppb in egg. Results from a fortified sample are compared to those from a control sample to show the differences in response.

DISCUSSION

Method Development. The current form of this multiclass extraction and analytical scheme evolved after a series of experiments with liquid/liquid, C-18 SPE, and cation exchange SPE procedures. First, it was shown that the C-18 SPE procedure developed for sulfonamides (*3*) could extract β-lactam drugs with detection limits at 5−10 ppb. Unfortunately, the tetracyclines were not recovered by this procedure. Various other procedures were then evaluated with tetracyclines and fluoroquinolones. Once the hydrophilic polymer Oasis HLB was tested, it became clear that this phase had a very broad

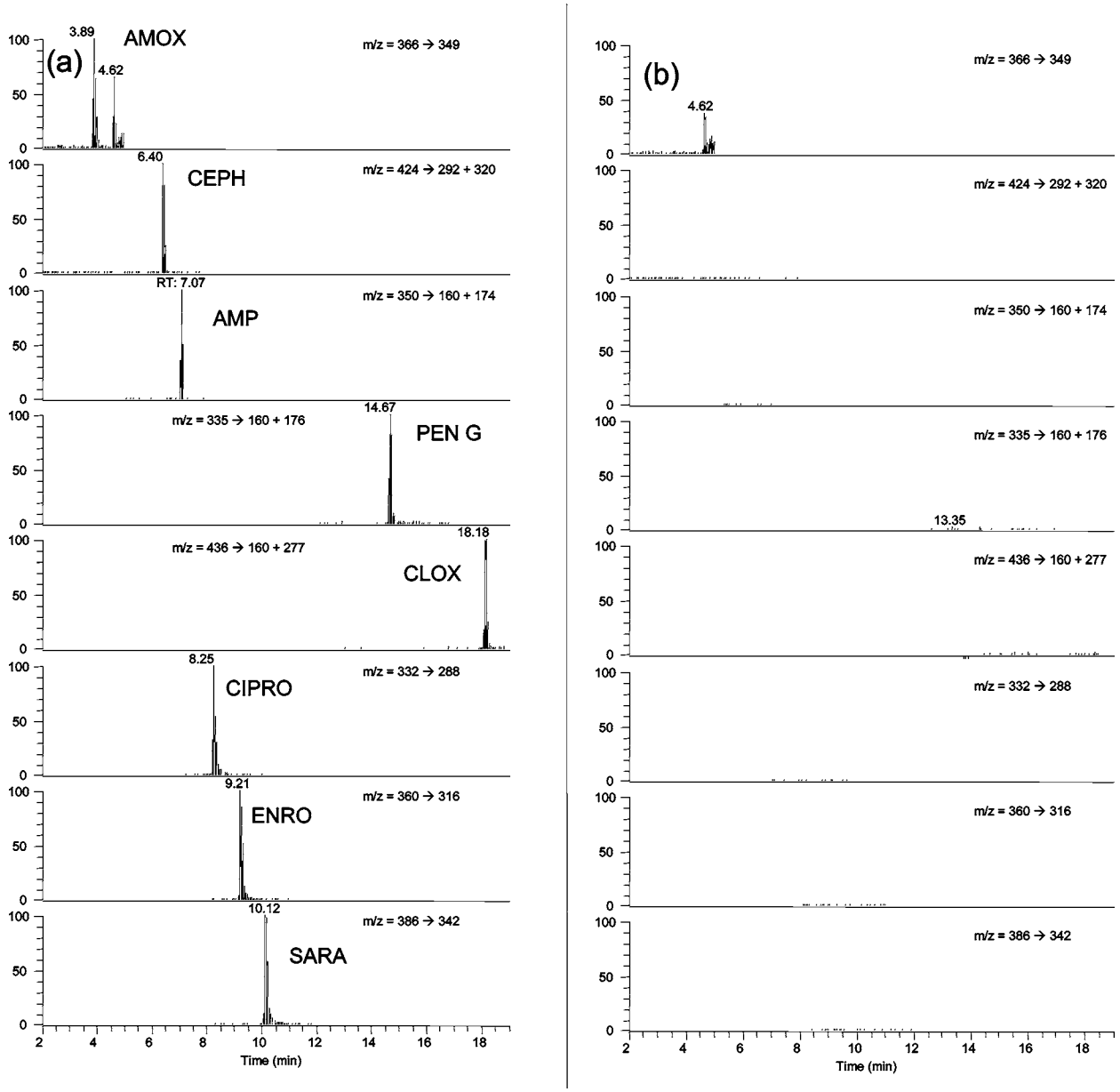

Figure 8. Multiclass screening analysis of drugs fortified in egg at 100 ppb: β-lactams and selected fluorquinolones (a), compared with a control egg extract (b), plotted to the same scale for each drug. Data were from the same extracts as in **Figure 7** (LCQ Classic).

applicability for polar compounds. Various solvents, buffer systems, and pH levels were tested before the method was refined to its present form.

Liquid Chromatography. The short, wide cartridge columns with 3 μm silica particles combine a high flow rate for faster gradients and fast equilibration with high chromatographic resolution (*35*). The LC gradient was run from high to low aqueous content to elute a wide variety of drug classes in a single run. However, the LC gradient cannot be so fast that it might compress multiple peaks in a close time frame, because this complicates data acquisition and increases the effect of interferences and matrix suppression. The phenyl column was chosen due to somewhat improved separation between various tetracyclines compared to that of a C-18 column.

Acquisition Methods. The primary reason for using data-dependent scanning was to maximize the duty cycle of scanning for an analyte that was actually detected. Optimum performance of the LCQ Classic called for "filling" the trap, i.e., storing a given ion flux for a sufficient time. For these compounds, the maximum isolation time was 500 ms, which meant that many seconds could elapse between scans for a given compound if the instrument iteratively searched for many compounds. In contrast, the optimum isolation time for the LCQ Deca XP Plus was only 25 ms.

Retention times and masses shown in **Table 1** were converted into the acquisition method shown in **Table 2**. It is worth noting that once a data-dependent method is created, target drug residues can be added or subtracted simply by adjusting the target mass list for two or three time segments. The method shown in **Table 2** includes four drug classes—tetracyclines, sulfonamides, fluoroquinolones, and β-lactams—to demonstrate the ability of this approach to handle nearly 30 compounds in one method.

Wideband activation refers to the performance of additional CID on first-stage product ions formed by loss of water or ammonia. Without wideband activation, four compounds produced only one MS^2 product ion following loss of NH_3: amoxicillin (*m*/*z* 366 → *m*/*z* 349), *N*-acetylsulfanilamide (*m*/*z* 215 → *m*/*z* 198), doxycycline (*m*/*z* 445 → *m*/*z* 428), and isochlortetracycline (*m*/*z* 479 → *m*/*z* 462). Wideband activation

Table 2. Acquisition Timing for the LCQ Classic Method

segment end time, min	isolation width, amu	analytes
3	1.6	SGD
4	5.0	SGD, AMOX
5	5.0	AMOX, *N*-AcSA
6	1.6	CEPH, *N*-AcSA, SDZ
7	5.0	CEPH, SDZ, AMP, SPD
8	5.0	CEPH, AMP, SDZ, SPD, OTC,[a] STZ, SMR, CIPRO, TC, SMZ
9	1.6	OTC, STZ, SMR, CIPRO, TC, SMZ, ENRO, SMPZ
10	1.6	CIPRO, TC, DMCC, ENRO, SMPZ, iso-CTC, SMTZ, SARA, DIFLOX
11	1.6	DMCC, iso-CTC, SMTZ, SARA, SMONO, DIFLOX, CTC, SCP, DOXY
12	1.6	DIFLOX, CTC, SCP, DOXY, SMXZ, SIX
13	5.0	SMXZ, SIX, SDM, PEN G, CLOX
14	5.0	SDM, SQX, PEN G, CLOX
15	5.0	SQX, PEN G, CLOX
16–19	5.0	PEN G, CLOX

[a] A time-scheduled MS^3 scan event was used for OTC in this segment to avoid an interference at *m*/*z* 461 that eluted at approximately the same time. Conditions were *m*/*z* 461.1 → *m*/*z* 443, isolation width 1.6, collision energy 23%, followed by *m*/*z* 443 → 426, isolation width 4, collision energy 28%.

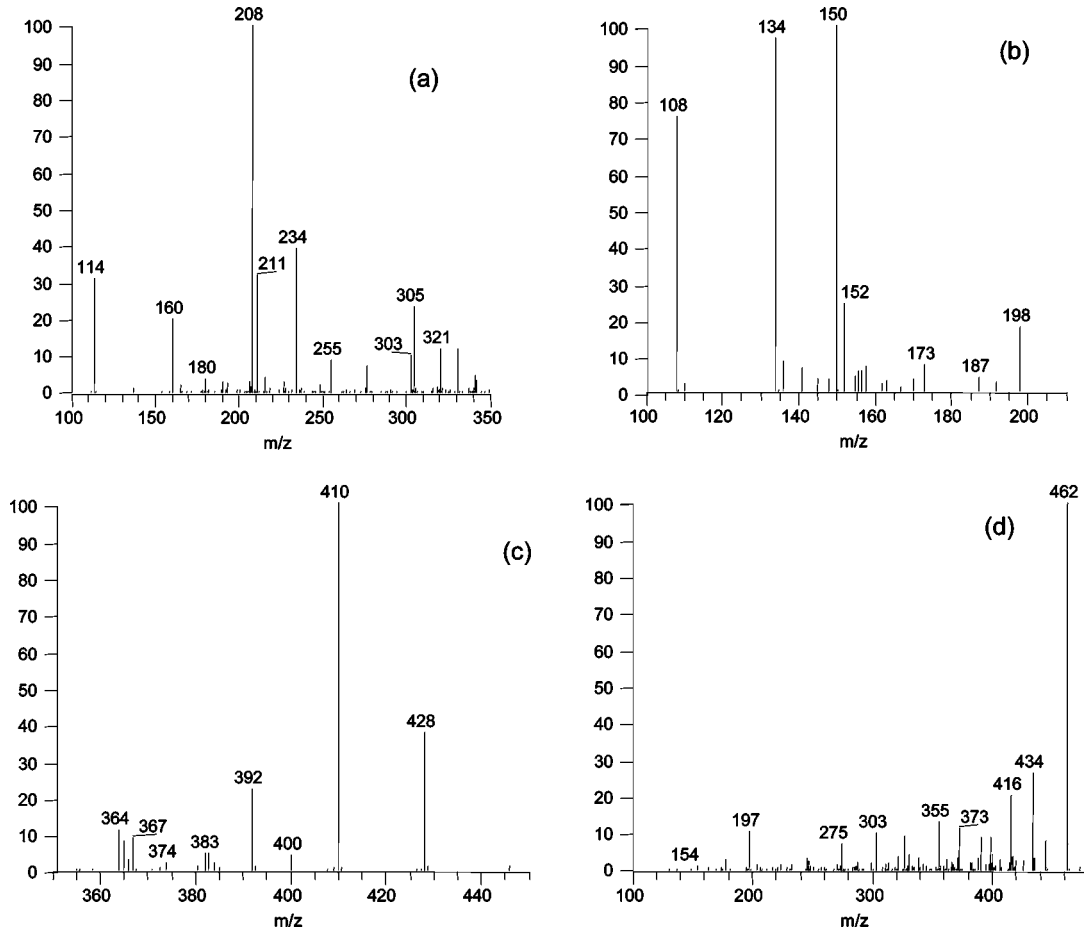

Figure 9. Mass spectra with enhanced fragmentation using wideband activation in MS^2 (LCQ Deca XP Plus): (a) amoxicillin, *m*/*z* 366 → *m*/*z* 349 →; (b) *N*-acetylsulfanilamide, *m*/*z* 215 → *m*/*z* 198 →; (c) doxycycline, *m*/*z* 445 → *m*/*z* 428 →; (d) isochlortetracycline, *m*/*z* 479 → *m*/*z* 462 →.

produced multiple third-generation ions in MS^2 mode as follows (**Figure 9**): amoxicillin, *m*/*z* 366 → *m*/*z* 349 → *m*/*z* 208 + *m*/*z* 234; *N*-acetylsulfanilamide, *m*/*z* 215 → *m*/*z* 198 → *m*/*z* 134 + *m*/*z* 150; doxycycline, *m*/*z* 445 → *m*/*z* 428 → *m*/*z* 410 + *m*/*z* 392; isochlortetracycline, *m*/*z* 479 → *m*/*z* 462 → *m*/*z* 434 + *m*/*z* 416.

For five other compounds, use of wideband activation in MS^2 still only yielded one major product ion, the "screen" ions shown in **Table 3**. Follow-up confirmatory analysis for these seven compounds would require both wideband activation and MS^3, as shown in **Table 3**.

Matrix Effects. When relatively dirty extracts are analyzed, coextractants build up on the column and begin to bleed off. Sometimes these provided an advantage. In particular, there was a matrix enhancement for the TC drug class when using the LCQ Classic. To take advantage of this phenomenon, one or more control extracts were injected prior to an analytical run to "prime" the system. On the other hand, PenG and CLOX

Table 3. MS^n Parameters for Follow-Up Confirmatory Analyses: MS^3 with Wideband Activation On

compound	MH^+	MS^2 screen ion, *m/z* (wideband activation on)	follow-up scan for confirmation	product ions
oxytetracycline	461.1	426	MS^3 of 426	337, 381
tetracycline	445.0	410	MS^3 of 410	154, 337
enrofloxacin	360.1	316	MS^3 of 360	245, 268
difloxacin	400.1	356	MS^3 of 356	299, 336
chlortetracycline	479.1	444	MS^3 of 444	154, 371

suffered matrix suppression more than other compounds (on both instruments). This issue could be partially overcome by changing the guard and analytical column.

For reasons that were not investigated fully, the LCQ Deca XP Plus interface did not exhibit the same favorable matrix enhancement, especially for tetracyclines. Similar method performance limits were observed for each instrument, suggesting that the presence of matrix coextractants is more of a controlling factor than fundamental instrument sensitivity. The enhanced sensitivity of the LCQ Deca XP Plus vs the LCQ Classic translated into shorter isolation times per scan, which in turn enabled time-scheduled acquisition for up to 10 coeluting compounds, more "normal" chromatographic peaks, simpler integration of chromatograms, and somewhat better ion statistics in the mass spectra.

Conclusions. Development of this method enabled us to explore the issues and challenges associated with multiclass residue analyses. Generic SPE cleanup and LC/MS analyses could accommodate a wide range of compounds, although there were tradeoffs. The recoveries of individual drug classes were lower than for a method that targets only one class. Conditions which maximized the response of a single product ion were preferable for screening purposes. Confirmatory analyses sometimes required conditions that divided analyte response among multiple diagnostic ions, resulting in decreased sensitivity. Even so, identification criteria for screening were consistently met at the target level of 100 ppb, meaning that this method can still serve its purpose.

The method was applied to eggs from dosed hens, and the ability to detect incurred residues of sulfonamides, tetracyclines, and fluoroquinolones was demonstrated. The method was not suitable for quantitation, although quantitation was performed of necessity to estimate the extraction efficiency and concentration range of incurred tissues. The method's performance on two generations of ion trap instruments seemed to be limited by coextractants more than by instrument sensitivity, especially when using compressed chromatography that led to increased matrix interference.

Ion trap tandem mass spectrometry is a flexible qualitative tool, although not offering the quantitative precision of triple-quadrupole mass spectrometers. Acquisition can be varied fairly easily from a basic framework by adding target ions to the search list, using wideband activation, or mixing data-dependent and time-scheduled acquisition. Such methods may necessarily exist in a state of flux as target compounds are added and newer instruments are put into service.

This study showed that multiclass methods are feasible for residue surveillance and explored factors that influence the success of such methods. It is still necessary to specify those analytes and performance criteria which are required for a given purpose and then to validate the specified method before regulatory application.

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