JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

A Novel Green Chemistry Method for Nonaqueous Extraction and High-Performance Liquid Chromatography Detection of First-, Second-, and Third-Generation Tetracyclines, 4-Epitetracycline, and Tylosin in Animal Feeds

Fabio Granados-Chinchilla,[†] Jorge Sánchez,[†] Fernando García,[‡] and César Rodríguez*[,‡]

[†]Centro de Investigaciones en Nutrición Animal (CINA), Universidad de Costa Rica, San José, Costa Rica

[‡]Centro de Investigación en Enfermedades Tropicales (CIET) and Facultad de Microbiología, Universidad de Costa Rica, San José 11501-2060, Costa Rica

ABSTRACT: Although tetracyclines and macrolides are common additives for animal nutrition, methods for their simultaneous determination in animal feeds are nonexistent. By coupling an organic extraction and solid-phase extraction cleanup to a high-performance liquid chromatography separation and a nonaqueous postcolumn derivatization, we succeeded in detecting from 0.2 to 24.0 μg kg^{-1} of tetracycline, oxytetracycline, chlortetracycline, doxycycline, tigecycline, and 4-epitetracycline in this complex and heterogeneous matrix. Minocycline and tylosin could also be detected with our procedure, but using UV spectrophotometry (1.5 $\leq$ LOD $\leq$ 1.9 mg kg^{-1}). Linear responses with correlation coefficients between 0.996 and 0.999 were obtained for all analytes in the 0.5−10 mg kg^{-1} concentration range. Average recoveries between 59 and 97% and between 98 and 102% were obtained for the tetracyclines and tylosin, respectively. Replicate standard deviations were typically below 5%. When this method was applied to 20 feeds marketed in Costa Rica, we detected labeling inconsistencies, banned mixtures of tetracyclines, and tetracycline concentrations that contravene international regulation.

KEYWORDS: *tetracyclines, tylosin, animal feed, HPLC, green chemistry*

INTRODUCTION

Because of their commercial availability and low cost, antibiotics from different families are added to animal feeds to enhance animal production.[1] In this context, antibiotic dosing must be carefully monitored to achieve the agronomic results that justify the use of these drugs, reduce the negative environmental and sanitary consequences of releasing antibiotics to agroecosystems,[2] prevent farmer exposure,[3,4] and protect the consumers.[5]

The tetracyclines (TCs) are among the most frequently and intensively used antibiotics in livestock production and veterinary medicine.[6] These compounds are unstable in solution,[7] and for this reason, their epimers are also subject to regulation.[8] A number of TCs and TC epimers can nowadays be determined in animal tissues, meats, and honey by reverse phase high-performance liquid chromatography (HPLC) of solid phase extracts, and although these compounds can be detected by ultraviolet light (UV), fluorescence, or mass spectrometry, the relatively poor sensitivity of UV, the high cost of LC-MS instruments, and the limitations in sample preparation and matrix effects linked to LC-MS[9] have favored the determination of fluorescence derivatives.

The performance of metal chelators such as Mg^{2+}, Al^{3+}, and Zr^{4+} in the derivatization of chlortetracycline (CTC), TC, doxycycline (DC), and oxytetracycline (OTC) has been studied,[10] and several buffers and pH conditions have been assayed to enhance the fluorescence of TCs chelates.[11] Methods capable of detecting down to 0.25 μg kg^{-1} of OTC and TC in muscle tissue by fluorescence are available;[12,13] however, they can still be improved regarding environmental, extraction, and sensitivity issues.

Tylosin (TY) is a macrolide employed in veterinary but not in human medicine. In some countries, this antibiotic is approved for the treatment of pneumonia and metritis in beef cattle,[14] erysipela, pneumonia, proliferative enteropathy, and dysentery in swine,[15] and respiratory diseases in poultry.[16] The detection of macrolides in food matrices is regularly achieved through UV-liquid chromatography[17,18] or electrochemical detection of extracts obtained with polymeric[19] or silica solid-phase extraction (SPE) cartridges.[20] The sensitivity of these methods is satisfactory.[21] However, they cannot detect other families of antibiotics, and their extraction steps are somewhat time-consuming.[18,19,22]

As it is desirable to detect multiple analytes in a single analysis[23] and bearing in mind that animal feeds contain antibiotics from different families, we developed a selective, accurate, and fast HPLC method for simultaneous determination of five first-, second-, and third-generation TCs, an epimer of TC, and tylosin in animal feeds. Besides the wide range of analytes covered, the proposed method is pioneering in coupling organic extraction to SPE[24] and later on to a nonaqueous derivatization system. The method can be fully accomplished in 12 min, and the amounts of methanol, dimethyl sulfoxide (DMSO), acetonitrile, and diluted trifluoro-

Received: January 3, 2012
Revised: June 25, 2012
Accepted: June 27, 2012
Published: June 27, 2012

ACS Publications © 2012 American Chemical Society

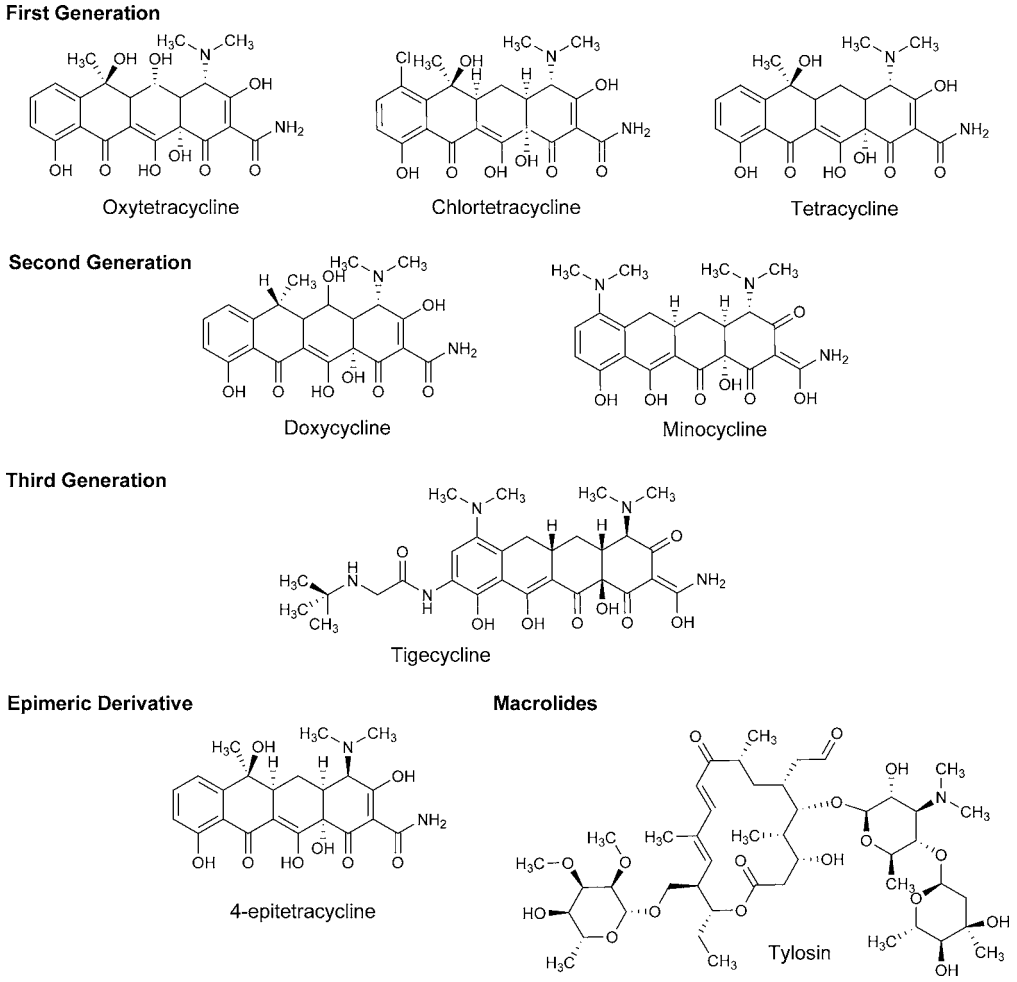

Figure 1. Chemical structure of the analytes covered by the proposed method. From top to bottom: first-generation TCs, second-generation TCs, third-generation TCs, an epimeric derivative of TC (4ET), and the macrolide tylosin.

acetic acid needed per run do not exceed 13 mL, converting it in an attractive green and safe technique.

Once validated, the method was applied to 20 commercial feed samples marketed in Costa Rica. The results of this survey are of sanitary and environmental relevance, as antibiotic dosing in feeds evades regulation in the developing world due to the lack of low-cost methods for multianalyte detection of antibiotics.

MATERIALS AND METHODS

Reagents and Materials. TC, OTC, CTC, minocycline (MC), doxycycline (DC), 4-epitetracycline (4ET), and TY standards, as well as dihydrate oxalic acid and trichloroacetic acid were purchased from Sigma-Aldrich. Tigecycline (TG) was obtained from Xingcheng Chempharm Co., Ltd. Acetonitrile and methanol were purchased from J. T. Baker Reagent Chemicals (HPLC grade). Water with a final conductivity of 0.055 μS/cm was obtained with a Millipore Milli-Q Advantage A10 system. SPE columns packed with hydrophilic–lipophilic balance (HLB) sorbent were obtained from Waters (Oasis, 33 μm, 60 mg, 3 mL). The chemical structures of the analytes detected by the proposed method are presented in Figure 1.

Preparation of Stock and Working Solutions of Standards. Stock standard solutions of 100 μg mL^{-1} were prepared by dissolving 2.5 ± 0.1 mg of TC, OTC, CTC, MC, DC, TG, 4ET, and TY in 25 mL of methanol. These solutions are stable for 6 months if stored below −18 °C in amber glass recipients.[7] Combined working standard solutions containing 10 to 0.5 μg mL^{-1} of the antibiotics were prepared by diluting stock solutions in methanol. These working solutions are stable for 1 month at 4 °C in the dark.[7]

Analyte Extraction and SPE Purification. Feed samples were sieved through a mesh opening size of 48 (0.297 mm). Five grams of sieved feed was then mixed with 20 mL of a methanolic solution containing 0.5 g citric acid and 2 mL nitric acid/100 mL. After homogenization for 1 min using an Ultraturrax (IKA T10 basic), the particles were separated from the solvent by centrifugation at 2500*g* for 10 min. The resulting pellets were mixed once again with 10 mL of the methanolic solution, and both extracts were pooled, centrifuged for 15 min at 2500*g*, and quantitatively transferred through Whatman 541 filters to 50 mL volumetric flasks that were filled up with methanol. These diluted extracts were loaded onto HLB columns that were preconditioned with 3 mL of methanol, 1 mL of 20 mM HCl, and 3 mL of water, in that order. Thereafter, the SPE columns were washed with 3 mL of 20 mM oxalic acid buffer (pH 4.0) and 2 mL of water and dried by applying vacuum for 10 min. The analytes were eluted with 4 aliquots of 500 μL of methanol.

Instrumentation. We used an Agilent 1200 series HPLC system equipped with a quaternary pump, fluorescence and variable wavelength detectors, autosampler, a column oven, a Zorbax SB C18 column (3 μm, 250 mm × 4.6 mm), and a temperated Pickering Laboratories Pinnacle PCX postcolumn derivatization system.

Chromatographic Conditions and Analyte Derivatization. A gradient of acetonitrile (A) and 20 mM trifluoroacetic acid in water (B) was used as a mobile phase at a flow rate of 0.7 mL min^{-1}: 0–10 min: 15% A, 85% B; 10–15 min: 60% A, 40% B. Fluorescent derivatives of TC, OTC, CTC, DC, TG, and 4ET were generated with DMSO, 1,4-dioxane, acetic acid, 2-ethoxyethanol (2-EE), ethyl acetate,

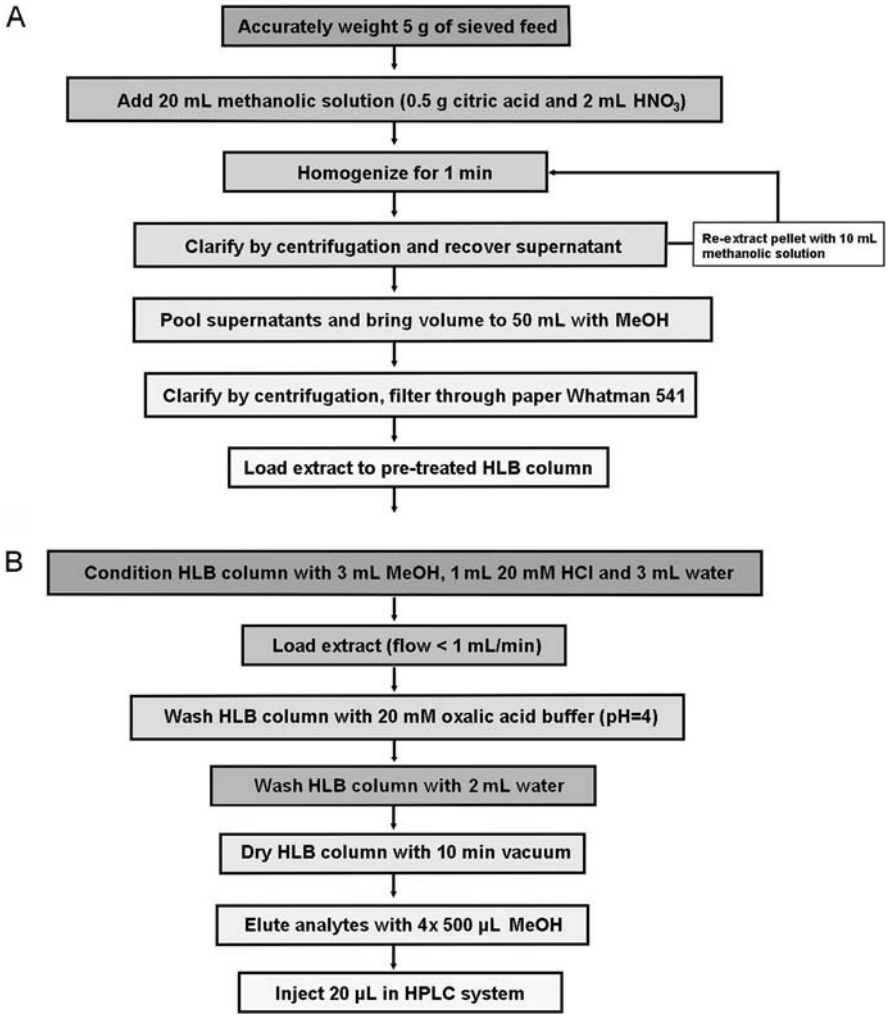

Figure 2. Proposed extraction (A) and cleanup procedure (B) for simultaneous HPLC determination of first-, second-, and third-generation TCs, 4ET, and tylosin in animal feeds.

N,*N*-dimethylformamide (DMF), *N*-dimethyl-2-pyrrolidone (DMP) or *N*,*N*-dimethylacetamide (DMAC), and 0.1 M magnesium acetate at a flow rate of 0.15 mL min^{-1} at 30 °C. These derivatives emit light at 473 nm after excitation at 399 nm. MC and TY were quantified with the UV detector at 355 and 280 nm, respectively. A diagram summarizing the final extraction and analytical procedure is presented in Figure 2.

Method Validation. To confirm that common feed components do not coelute with the analytes, seven nonmedicated feeds from LGC and the Association of American Feed Control Officials (AAFCO), as well as certified feeds from the Animal Feeds Scheme (AFPS), were analyzed with the proposed method. The accuracy was measured as the percent of deviation of results obtained for seven standard solutions of known concentration. Repeatability and intermediate precision were investigated by assaying blank fish feed samples spiked at the 0.05, 1, 50, 200, and 400 mg kg^{-1} levels 10 times on a single day and on different days. Linearity was evaluated by means of regressions relating peak areas to analyte concentrations in the 0.5−10 mg kg^{-1} range. Limits of detection (LOD) and limits of quantification (LOQ) were defined as the analyte concentrations that could be detected with signal-to-noise ratios (S/N) > 3.3 or that yielded peaks with S/N > 10, respectively. S/N values were obtained by lineal regression simple precision calculation (i.e., $3s/m$) and blank feed baseline inspection. LODs were corroborated with the lowest spike level. Recoveries were determined with 10 samples of unmedicated fish feed to which different concentrations of the analytes were added before extraction. With the exception of TG and 4ET, fortification levels of 0.05, 1, 50, 200, or 400 mg kg^{-1} were tested for the TCs. These spike levels where chosen based on the concentrations of OTC and CTC in animal feeds allowed by the Food and Drug Administration (FDA).[24] The recovery of TG and 4ET was determined with less spike levels because TG is not expected to be present in animal feeds and because 4ET—if present at all—should only be found in feeds that were stored for long periods of time. The TY recovery was determined using fortification levels of 50 and 900 mg kg^{-1}. To ensure proper analyte identification and adequate performance of the chromatographic system, retention times (R_T) ± 3 standard deviations, number of theoretical plates (N), and tailing factors (T_f) were determined for 10 blank feed samples spiked with 50 mg kg^{-1} of each antibiotic.

Statistical Analyses. Associations between the dielectric constants of the solvents and the fluorescence of the derivatives were assessed by means of Spearman's correlation coefficients. In addition, a Kruskal−Wallis test was employed to evidence differences in the fluorescence intensity of derivatives prepared with various solvents.

Application of the Method to Commercial Feed Samples. The method depicted in Figure 2 was applied to feed for diverse growing stages of shrimp (n = 7), tilapia (n = 5), swine (n = 4), and poultry (n = 4) collected by government inspectors in 15 Costa Rican mills. To avoid analyte degradation, the field samples were stored at 2 °C in a dry and dark environment for a maximum of 30 days before analysis. A medicated starter feed for suckling pigs obtained from AAFCO was analyzed in parallel to verify the accuracy of the method (check sample no. 201021).

RESULTS AND DISCUSSION

While others use the aqueous-based McIlvaine/ethylenediaminetetraacetic acid (EDTA) buffer at pH 4 to extract TCs,[25] we preferred a methanolic solution containing citric and nitric acid to eliminate the phase separation and filtration steps associated with the former buffer. This nonaqueous extraction is advantageous because the TCs are more soluble in methanol than in water[25,26] and also because methanol facilitates solvation of calcium and magnesium ions, which are found in relatively high concentrations and are bound to other components of the animal feeds[27,28] and interfere in the determination of TCs.[29] These divalent cations were removed with the cost-effective chelating agent citric acid, and nitric acid was added to overcome the propensity of methanol to extract excessive matrix material[25] and to denature proteins and nucleic acids that could interact with the analytes. Addition of >0.5 g of citric acid to 50 mL of the methanolic extraction solution did not improve the recovery rates of five of the TCs covered by the method (data not shown). On the other hand, elimination of citric and nitric acid from the extraction buffer drastically reduced recoveries, whereas the addition of more than 2 mL nitric acid/100 mL extraction solution was unnecessary (data not shown).

The pH of the extraction solution was kept low to protonate the secondary amine group at position 4 of the A ring of the TCs and thereby promote their binding to the *N*-vinylpyrrolidone moiety of the HLB stationary phase. Furthermore, this low pH increases the solubility of tylosin in methanol due to protonation of the 4-dimethylamino group in its pyranose ring (pK_a = 7.7). HLB SPE columns were preferred because their sorbent retains both polar and nonpolar drugs without collapse at low pH and also because they do not exhibit the silanol activity of some C-18 SPE cartridges.[30]

With few exceptions, the TCs with the lowest LOD showed the poorest recoveries (Table 1). The average recoveries of OTC (62.3%) and TC (74.4%) were much lower than those determined for CTC (94.5%) and DC (94.6%), whereas the average recovery of MC was intermediate (83.2%). In opposition to its low LOD, the average spike recovery of tylosin was particularly high (>98%) (Table 2).

As the most polar TCs (OTC and TC) could be lost during washing of the SPE columns, we tested whether replacement of water by solvents of lower polarity would improve their recoveries. Cartridge cleanup with ethyl acetate or with methanolic 10 mmol/L oxalic acid and ethyl acetate (1:3) improved the recovery of OTC in ca. 20%. However, we did not adopt this modification to maintain a one-step-one-solvent extraction method and also because it did not improve the recovery of other TCs. We assume that the lower losses of MC in the SPE cleanup are due to its two amine groups, as they have the potential to strongly interact with the HLB sorbent upon protonation. On the other hand, the chlorine atom in CTC, which turns this molecule into a very efficient Lewis acid and a strong chelating agent, may be the cause of its slow extraction from the feeds (data not shown).

The retention times of the TCs loosely correlated with their octanol–water partitions: MC (log K_{ow} = −1.93, RT = 4.46), TC (log K_{ow} = −0.890, RT = 6.42), OTC (log K_{ow} = −1.131, RT = 7.27), 4ET (log K_{ow} = −1.2, RT = 8.52), CTC (log K_{ow} = −0.360, RT = 9.44), and DC (log K_{ow} = −0.220, RT = 10.06). Despite the strong structural similarity of TC and its reversible epimer 4ET, our gradient elution separated both compounds satisfactorily (Figure 3B,C). TY (log K_{ow} = 1.630, RT = 11.81) eluted after acetonitrile reached its maximum concentration in the mobile phase (Figure 3D). Tailing factors for all analyte responses invariably lied below 2, theoretical plates ranged from 4 × 10^3 to 13 × 10^3, and capacity factors (k') oscillated between 1.51 and 2.65. No interferents were observed in the elution regions of the analytes (Figure 3F).

The TCs emit fluorescence due to their β-diketone system at positions 11 and 12 of the C ring, enol groups at positions 1 and 3, and the carboxamide at position 2 of the A ring.[26,31] Aqueous buffers based on boric acid have been traditionally used to yield fluorescent derivatives of TCs.[10,12,13] However, stronger signals for 4-ET, OTC, TC, CTC, and DC have been obtained in recent times with DMF and DMAC in combination

Table 1. Recovery of TCs with the Proposed Method

analyte	spike level (mg kg^{-1})	average recovery (%)[a]	standard deviation[a]
OTC	0.05	60.1	6.3
	1	61.2	3.1
	50	68.8	6.5
	200	63.0	4.9
	400	59.4	4.3
TC	0.05	78.2	11.2
	1	74.7	3.9
	50	81.0	4.5
	200	70.0	3.5
	400	72.3	4.8
CTC	0.05	82.8	7.2
	1	86.3	1.4
	50	93.3	3.2
	200	92.4	4.0
	400	97.7	2.5
DC	0.05	91.5	13.0
	1	88.6	3.3
	50	95.6	4.4
	200	96.4	1.0
	400	92.0	3.0
MC	5	84.1	3.5
	50	87.6	4.1
	200	80.8	5.7
	400	81.3	4.2
4ET	0.05	89.8	12.1
	1	88.8	1.4
	50	78.2	7.82
TG	0.05	80.2	10.6
	1	83.4	4.6
	50	89.8	2.2

[a]Mean of 10 replicates.

Table 2. Recovery of Tylosin with the Proposed Method

analyte	spike level (mg kg^{-1})	average recovery (%)[a]	standard deviation (%)[a]
TY	50.2	102.8	3.07
	907	98.6	1.48

[a]Mean of 10 replicates.

dx.doi.org/10.1021/jf301403f | J. Agric. Food Chem. 2012, 60, 7121−7128

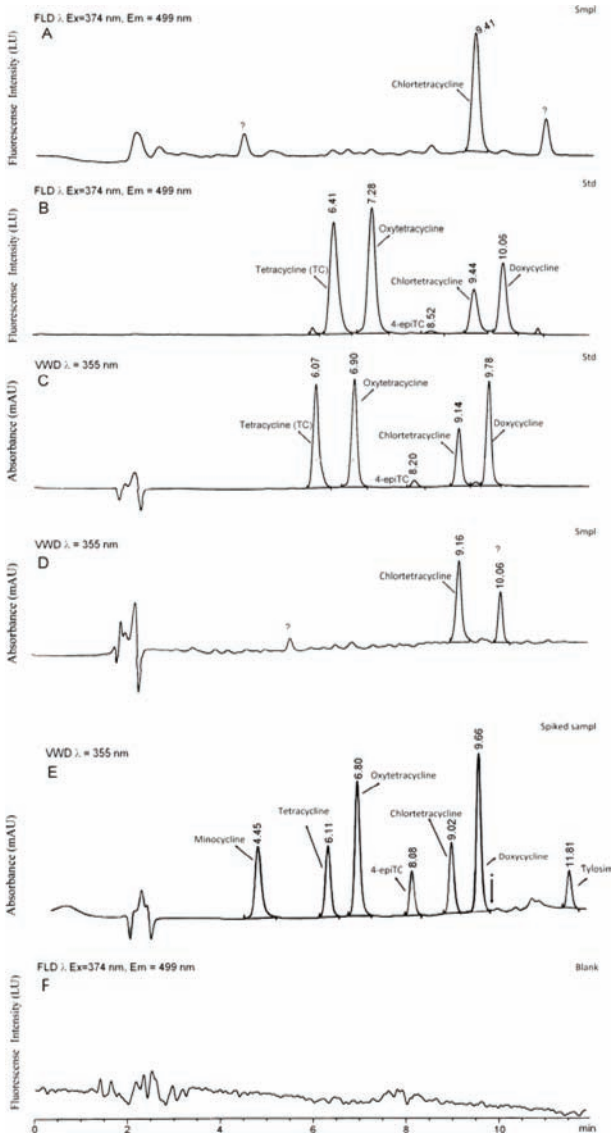

Figure 3. (A) Detection of CTC in reference sample P-964 with the proposed HPLC method. (B) Fluorescence detection of a mixture of TCs in methanol at a final concentration of 500 ng mL^{-1} each. (C) UV detection of a mixture of TCs in methanol at a final concentration of 500 ng mL^{-1} each. (D) UV detection of CTC in a sample of fish feed. (E) UV analysis of a fish feed spiked with 20 μg of each analyte. The vertical arrow at 10 min indicates a wavelength shift from 355 to 285 nm to allow detection of tylosin. (F) Chromatogram of an unmedicated pig starter feed sample.

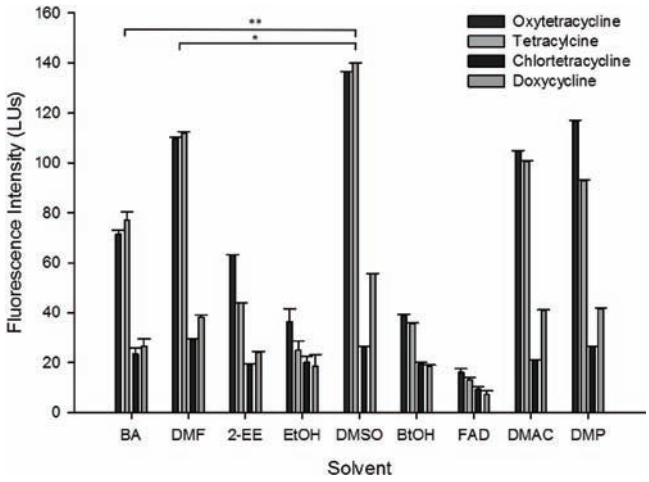

Figure 4. Fluorescence intensity of postcolumn derivatives of four TCs generated with DMSO (46.6 D), DMAC (37.8 D), DMF (36.7 D), DMP (32.2 D), EtOH (22.4 D), BtOH (16.56 D), FAD (2.4 D), and 2-EE (5.3 D). The dielectric constants of these solvents at 20 °C are shown in parentheses. Bars represent the mean of three replicates, and error bars depict standard deviations. The fluorescence obtained with DMSO was significantly higher than that obtained with aqueous buffer (BA) (**, $p < 0.001$) or with DMF (*, $p < 0.01$).

with 0.01 mol L^{-1} oxalic acid.[11] From all of the solvents used for derivatization, DMSO gave rise to the strongest signals, and derivatives of OTC and TC exhibited the strongest fluorescence overall (Figure 4). No fluorescence was observed when the TCs were derivatized with 1,4-dioxane, acetic acid, or ethyl acetate, and unspecific signals were obtained with ethanol (EtOH). Interestingly, the fluorescence of the derivatives and the dielectric constants of DMF, DMSO, DMP, and DMAC were negatively related (Spearman's ρ: −0.855, $p < 0.01$), possibly due to solvatochromic shifts.[32] The superiority of DMSO could be a consequence of its high dipole moment and capacity to promote the extended conformation of TCs.[31] In addition, DMSO enhances the acid−base properties of acetate and promotes the complexation of deprotonated TCs with magnesium,[33] and the solubility of Mg^{2+} acetate in DMSO is sufficient to immediately generate complexes with the amounts of TCs expected in the samples. Moreover, DMSO is less toxic than DMF, DMP, or DMAC, it can be degraded by biological systems,[34,35] and it is highly soluble in water, diminishing the chance of crystal formation in the HPLC tubing. Further work should be done to control undesired reactions that could diminish the sensitivity of our assay, such as reversed-coordination between magnesium and acetate during derivatization.[36]

In ascending order of sensitivity, OTC, TC, CTC, DC, and TG were detected by fluorescence down to μg kg^{-1} levels (Table 3). 4ET could also be detected by fluorescence, but its LOD was approximately 100 times higher than that obtained for other TCs (Table 3). MC is also a TC, but it did not emit fluorescence under our experimental conditions perhaps due to some degree of structural hindrance during chelation or to the high electron donor capability of its amine group in carbon atom 7 of the ring D.[37] Nonetheless, MC—as any other TC—absorbs light in the 350−355 nm range. We have achieved μg kg^{-1} sensitivities for MC and 4ET, but this improvement requires elution with a concentration of oxalic acid that could lead to some degree of precipitation (>50 mmol/L; data not shown).

LODs of MC and TY were in the mg kg^{-1} range (Table 3). TY does not emit fluorescence probably due to its lack of rigidity. However, it has a relatively strong absorption peak at 290 nm that enables its detection by UV. As some feeds contain urea phosphate and urea may react with TY to produce double peaks in HPLC-UV/DAD analyses,[38] the temperature of the column oven was maintained at 45 °C. A diode array detector and a different solid extraction sorbent could be considered to lower the detection limit obtained for this macrolide.

All analytes exhibited linear responses in the 0.5−10 mg kg^{-1} concentration range with correlation coefficients ranging from 0.9965 to 0.9999 (Table 3). Replicate standard deviations (RSDs) were typically below 5%, except for the lower spike level (Table 1). DC exhibited the lowest RSD overall (1−3% at

Table 3. Linearity, LODs, and LOQs of Five TCs, One Epimer of TC, and Tylosin with a Multianalyte HPLC Method

analyte	detection	linear regression	Pearson's coefficient	LOD/LOQ (μg kg^{-1} or mg kg^{-1})[a]
TC	fluorescence	$y = (34.3 \pm 0.1)x - (47.5 \pm 3.2)$	0.9999	0.2/0.6
OTC		$y = (32.3 \pm 0.2)x - (17.9 \pm 2.0)$	0.9999	0.5/1.5
CTC		$y = (22.3 \pm 0.2)x - (44.9 \pm 2.9)$	0.9999	0.2/0.6
DC		$y = (27.1 \pm 0.3)x - (11.6 \pm 1.1)$	0.9987	0.5/1.5
TG		$y = (28.2 \pm 0.3)x + (10.3 \pm 1.3)$	0.9996	7.8/23.6
4ET		$y = (15.7 \pm 0.5)x - (1.0 \pm 0.7)$	0.9998	24.0/72.7
MC	UV	$y = (31.3 \pm 0.4)x - (40.1 \pm 3.2)$	0.9996	0.5/1.5
TY		$y = (25.7 \pm 0.4)x - (3.8 \pm 1.0)$	0.9965	1.9/5.6

[a] μg kg^{-1} for all TCs except MC and mg kg^{-1} for compounds detected with UV spectrophotometry.

Table 4. Application of the Proposed Multianalyte HPLC Method to Samples of Animal Feed Marketed in Costa Rica

		analytes detected (mg kg^{-1})[c,d]				
sample[a]	antibiotic reported in the label (mg kg^{-1})[b]	CTC	TC	OTC	DC	result interpretation[e]
P-964[f]	CTC (364.0)	357.1				
P-967	none	501.7	84.7	68.1	68.0	1
P-968	CTC (100)	126.1	70.8			3
P-969	none	124.8			21.1	1
P-970	CTC (230)			27.1		2, 4
P-971	none	229.0	182.0	196.9	168.7	1, 2
C-972	none	34.7				1
C-973	OTC (200)	98.7	33.8	60.1		2, 4
C-974	NR			487.8		1
C-975	OTC (50), DC (NR), TY (50)	197.5	127.1	69.7	66.6	2, 3, 4
F-976	none	90.1	84.0	57.6	34.5	1, 2
F-977	OTC (120)			90.5		4
F-978	none			2070.0		1
F-979	none		94.5	2246.2	26.7	1, 2
F-980	none	40.1	37.2	134.5	16.4	1, 2
F-981	none			2014.2		1
S-1000	none	164.8	68.9			1, 2
S-1001	none	28.8		12.5		1, 2
S-1002	none			143.3		1, 2
S-1003	none	51.7			44.7	1, 2
S-1006	none	114.2				1, 2
S-1008	none	24.8	3.4			1, 2

[a] P, swine feed; C, poultry feed; F, fish feed; and S, shrimp feed. [b] NR, not reported. [c] The results are expressed as the mean of three replicates. Standard deviations were invariably below 10%. [d] Tylosin was not detected in any of the samples. [e] Result interpretation: 1, antibiotic detected in nonmedicated feed; 2, unexpected antibiotics detected; 3, concentration detected > concentration reported; and 4, concentration detected < concentration reported. [f] Sample 964 is check sample no. 201021 from the Association of American Feed Control Officials. $z_{\sigma=38.1}$ values <1 were obtained for this control sample after duplicate analysis in three different occasions.

the 200 and 400 mg kg^{-1} spike levels), and the RSD of tylosin was satisfactory (<3%).

Up to 67% of the commercial samples were labeled as nonmedicated contained antibiotics in concentrations as high as 2246.2 mg kg^{-1} (Table 4). Two out of 10 medicated samples had more TCs than expected, and 13 samples contained mixtures of TCs in amounts that defy FDA guidelines (Table 4). On the other hand, three samples had lower amounts of antibiotics than claimed in the labeling (Table 4), and none of the samples contained detectable amounts of tylosin. These results agree with previous reports.[39] If a pig of 110 kg and 22 weeks of age was fed with 4000 g of sample P-971 every day, it would ingest 30 mg TCs kg^{-1} of live weight every 24 h, three times the usual dose for bacterial enteritis.[40] In another example, a 220 g tilapia fish eating 7 g of sample F-978 two or three times a day would almost exceed, in a single feeding, the upper limit of 18.8 mg of OTC per harvested fish authorized by the FDA. We did not find MC or TG in the samples, but this was expected because they are only meant for human use.[41]

As the analytes eluted within 12 min with a relative low flow rate of the mobile phase and a solvent gradient, a modest volume of solvent waste was generated, and usage of acetonitrile and trifluoroacetic acid was minimal. On top of that, because each run only requires 10.5 mL of mobile phase—including <2 mmol of trifluoroacetic acid and 3.15 mL of the high-priced solvent acetonitrile—our procedure emerges as an environmentally friendly, safe, and economical alternative to current methods. We did not substitute TFA with an organic salt such as oxalic acid to limit precipitation in the system tubing, and methanol and acetonitrile were used because they are also considered green solvents[29] and are used in relatively low quantities. Moreover, methanol and citric acid are naturally occurring compounds. Summarizing, we developed a fast, sensitive, safe, and eco-friendly multicomponent analysis for

some of the most commonly used antibiotics in animal feedstuff. It is simple and can be performed with instrumentation accessible in the developing world.

AUTHOR INFORMATION

Corresponding Author

*Phone: (+506) 2511-4364. E-mail: cesar.rodriguezsanchez@ucr.ac.cr.

Funding

This work was supported by CONARE through a FEES grant.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to Gloriana Villalta for her technical assistance, Alfonso García Piñeres for his valuable input to a preliminary version of this manuscript, and the companies that facilitated the feed samples.

ABBREVIATIONS USED

SPE, solid-phase extraction; UV, ultraviolet light; FDA, Food and Drug Administration; HLB, hydrophilic−lipophilic balance; HPLC, high-performance liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; OTC, oxytetracycline; CTC, chlortetracycline; DC, doxycycline; TC, tetracycline; 4ET, 4-epitetracycline; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; FLD, fluorescence detector; 2-EE, 2-ethoxyethanol; EtOH, ethanol; BtOH, *tert*-butanol; FAD, formaldehyde; DMAC, *N*,*N*-dimethylacetamide; DMP, *N*-dimethyl-2-pyrrolidone; EDTA, ethylenediaminetetraacetic acid.

REFERENCES

(1) Bloom, R. A. Use of veterinary pharmaceuticals in the United States. In *Pharmaceuticals in the Environment: Sources, Fate, Effects, and Risks*; Kummerer, K., Ed.; Springer-Verlag: Germany, 2004; pp 154−158.

(2) Du, L.; Liu, W. Occurrence, fate, and ecotoxicity of antibiotics in agro-ecosystems. A review. *Agron. Sustainable Dev.* **2012**, *32*, 309−327.

(3) Hamscher, G.; Pawelzick, H. T.; Sczesny, S.; Nau, H.; Hartung, J. Antibiotics in dust originating from pig-fattening farm: a new source of health hazard for farmers? *Environ. Health Perspect.* **2003**, *111*, 1590−1594.

(4) Wegener, H. C.; Aaresturup, F. M.; Gerner-Smidt, P.; Bager, F. Transfer of antibiotic resistant bacteria from animals to man. *Acta Vet. Scand. Suppl.* **1999**, *92*, 51−58.

(5) Annual Report 2009 on the Rapid Alert System for Food and Feed, http://ec.europa.eu/food/food/rapidalert/docs/report2009_en.pdf. Date of last visit: 02/27/2012).

(6) Gilchrist, M. J.; Greko, C.; Wallinga, D. B.; Beran, G. W.; Riley, D. G.; Thorne, P. S. The Potential Role of Concentrated Animal Feeding Operations in Infectious Disease Epidemics and Antibiotic Resistance. *Environ. Health Perspect.* **2007**, *115*, 313−316.

(7) Liang, Y.; Denton, M. B.; Bates, R. B. Stability studies of tetracycline in methanol solution. *J. Chromatogr., A* **1998**, *827*, 45−55.

(8) Gadja, A.; Posyniak, A. Tetracyclines and their epimers in animal tissues by high-performance liquid chromatography. *Bull. Vet. Inst. Pulawy.* **2009**, *53*, 263−267.

(9) Cappiello, A.; Famiglini, G.; Palma, P.; Pierini, E.; Termopoli, V.; Trufelli, H. Overcoming Matrix Effects in Liquid Chromatography-Mass Spectrometry. *Anal. Chem.* **2008**, *80*, 9343−9348.

(10) Granados, M.; Encabo, R.; Compañó, R.; Prat, M. D. Determination of Tetracyclines in Water Samples Using Liquid Chromatography with Fluorimetric Detection. *Chromatographia* **2005**, *61*, 471−477.

(11) Spisso, B. F. Validation of a high-performance liquid chromatographic method with fluorescence detection for the simultaneous determination of tetracyclines residues in bovine milk. *Anal. Chim. Acta* **2007**, *581*, 108−117.

(12) Peña, A. L.; Lino, C. M.; Silveira, M. I. Determination of Tetracycline Antibiotics in Salmon Muscle by Liquid Chromatography Using Post-Column Derivatization with Fluorescence Detection. *J. AOAC Int.* **2003**, *86*, 925−929.

(13) Peña, A.; Pelantova, C. M.; Lino, C. M.; Silveira, M. I.; Solich, P. Validation of an Analytical Methodology for Determination of Oxytetracycline and Tetracycline Residues in Honey by HPLC with Fluorescence Detection. *J. Agric. Food Chem.* **2005**, *53*, 3784−3788.

(14) Apley, M. D.; Coetzee, J. F. Antimicrobial use in Cattle. In *Antimicrobial Therapy in Veterinary Medicine*; Giguère, S., Prescott, J. F., Baggot, J. D., Waker, R. D., Dowling, P. M., Eds.; Blackwell Publishing: Gaithersburg, MD, 1995; pp 485−506.

(15) Friendship, R. M. Antimicrobial use in Swine. In *Antimicrobial Therapy in Veterinary Medicine*; Giguère, S., Prescott, J. F., Baggot, J. D., Waker, R. D., Dowling, P. M., Eds.; Blackwell Publishing: Gaithersburg, MD, 1995; pp 535−544.

(16) Hofacre, C. L. Antimicrobial use in Poultry. In *Antimicrobial Therapy in Veterinary Medicine*; Giguère, S., Prescott, J. F., Baggot, J. D., Waker, R. D., Dowling, P. M., Eds.; Blackwell Publishing: Gaithersburg, MD, 1995; pp 545−550.

(17) McGlinchey, T. A.; Rafter, P. A.; Regan, F.; McMahon, G. P. A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices. *Anal. Chim. Acta* **2008**, *624*, 1−15.

(18) Dreassi, E.; Corti, P.; Bezzini, F.; Furlanetto, S. High-performance liquid chromatographic assay of erythromycin from biological matrix using electrochemical or ultraviolet detection. *Analyst* **2000**, *125*, 1077−1081.

(19) González de la Huebra, M . J.; Vincent, U.; von Holst, C. Sample preparation strategy for the simultaneous determination of macrolide antibiotics in animal feedingstuffs by liquid chromatography with electrochemical detection (HPLC-ECD). *J. Pharm. Biomed. Anal.* **2007**, *43*, 1628−1637.

(20) Heller, D. N.; Nochetto, C. B. Development of multiclass methods for drug residues in eggs: silica SPE cleanup and LC-MS/MS analysis of ionophore and macrolide residues. *J. Agric. Food Chem.* **2004**, *52*, 6848−6856.

(21) Wang, J.; Leung, D. Analyses of macrolide antibiotic residues in eggs, raw milk, and honey using both ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry and high-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3213−3222.

(22) Civitareale, C.; Fiori, M.; Ballerini, A.; Brambilla, G. Identification and quantification method of spiramycin and tylosin in feedingstuffs with HPLC−UV/DAD at 1 ppm level. *J. Pharm. Biomed. Anal.* **2004**, *36*, 317−325.

(23) Casewell, M.; Friis, C.; Marco, E.; McMullin, P.; Phillips, I. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* **2003**, *52*, 159−161.

(24) U.S. Food and Drug Administration (FDA). Web site. Available at www.accessdata.fda.gov/drugsatfda_docs/label/2011/050808s014lbl.pdf. Accessed June 24, 2012.

(25) Anderson, C. R.; Rupp, H. S.; Wu, W.-H. Complexities in tetracycline analysis—Chemistry, matrix extraction, cleanup, and liquid chromatography. *J. Chromatogr., A* **2005**, *1075*, 23−32.

(26) Zakeri, B.; Wright, G. D. Chemical Biology of tetracyclines. *Biochem. Cell. Biol.* **2008**, *86*, 124−136.

(27) Eisemann, J. H.; van Heugten, E. Response of pigs to dietary inclusion of formic acid and ammonium formate. *J. Anim. Sci.* **2007**, *85*, 1530−1539.

(28) Bouravela, I.; Chagneaub, A.; Lecuellea, S.; Lescoatb, P.; Ferreirac, G.; Duvaux-Ponterd, C.; Leterrierc, C. Feed composition and hardness interact in preference and intake in chickens. *Appl. Anim. Behav. Sci.* **2009**, *118*, 62−68.

(29) Capello, C.; Fischer, U.; Hungerbühler, K. What is a green solvent? A comprehensive framework for the environmental assessment of solvents. *Green Chem.* **2007**, *9*, 927−934.
(30) Blahová, E.; Brandšteterová, E. Approaches in Sample Handling before HPLC Analysis of Complex Matrices. *Chem. Pap.* **2004**, *58*, 362−373.
(31) Jameson, L. P.; Dzyuba, S. V. Spectroscopic Studies on Tetracycline in Room-Temperature Ionic Liquids. *J. Nat. Prod.* **2011**, *74*, 310−313.
(32) Mataga, N.; Kaifu, Y.; Koizumi, M. The solvent effect on fluorescence spectrum. Change of solute-solvent interaction during the lifetime of excited solute molecule. *Bull. Chem. Soc. Jpn.* **1955**, *28*, 690−691.
(33) Bordwell, F. Equilibrium acidities in dimethyl sulfoxide solution. *Acc. Chem. Res.* **1988**, *21*, 456−463.
(34) Brown, V. K.; Robinson, J.; Stevenson, D. E. A note on the Toxicity and Solvent Properties of DMSO. *J. Pharm. Pharmacol.* **1963**, *15*, 688−692.
(35) Brayton, C. P. DMSO—A review. *Elf. Aquitaine. Lacq.* **1986**, *76*, 61−90.
(36) Hojo, M. Elucidation of specific ion association in nonaqueous solution environments. *Pure Appl. Chem.* **2008**, *80*, 1539−1560.
(37) Wessels, J. M.; Ford, W. E.; Zsymczak, W.; Schneider, S. The Complexation of Tetracycline and Anhydrotetracycline with Mg^{2+} and Ca^{2+}: A Spectroscopic Study. *J. Phys. Chem. B* **1998**, *102*, 9323−9331.
(38) Gramse, M. J.; Jacobson, P. E.; Selkirk, J. C. Determination of tylosin in feeds by liquid chromatography with solid-phase extraction. *J. AOAC Int.* **2004**, *87*, 341−345.
(39) Gutiérrez, K.; Alfaro, M.; Granados, F.; Sánchez, J.; García, F.; Rodriguez, C. Microbiological detection of tetracyclines in 9 lots of animal feed produced in Costa Rica: Infringement of norms and incongruences with the official labeling. *Agron. Costarricense.* **2010**, *34*, 145−151.
(40) Kilroy, C. R.; Hall, W. F.; Bane, D. P.; Bevill, R. F.; Koritz, G. D. Chlortetracycline in swine bioavailability and pharmacokinetics in fasted and fed pigs. *J. Vet. Pharmacol. Ther.* **1990**, *13*, 49−58.
(41) Grolman, D. C. Therapeutic applications of tigecycline in the management of complicated skin and skin structure infections. *Int. J. Infect. Dis.* **2007**, *11*, S7−S15.