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Development and Validation of a Multiclass Method for the Analysis of Veterinary Drug Residues in Eggs Using Liquid Chromatography-Tandem Mass Spectrometry

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Abstract A liquid chromatography tandem-mass spectrometry (LC-MS/MS) method has been developed for the simultaneous determination of 22 veterinary drug residues, belonging to eight classes (coccidiostats, lincosamides, macrolides, tetracyclines, sulfonamides, benzimidazoles, diterpenes, and diaminopyrimidines), in eggs. Chromatographic separations were achieved on an XBridge BEH C18 column (150 × 2.1 mm, 3.5 μm, Waters, USA) maintained at 35 °C. The mobile phase was eluted at 400 μL min^{-1} in gradient mode between water and methanol/acetonitrile (20:80 v/v), both containing 0.1 % formic acid. The samples were prepared by protein precipitation with acetonitrile without additional cleanup steps. The method was successfully validated according to the Commission Decision 2002/657/EC and was demonstrated to be highly selective and free of matrix and residual effects. The method presented low limits of detection (0.37 to 7.5 μg kg^{-1}) and quantification (1.25 to 20 μg kg^{-1}). For banned substances, the decision limit values (CCα) and detection capability (CCβ) were 0.62–7.5 and 0.65–8.1 μg kg^{-1}, respectively. For substances with a maximum residue limit, the CCα and CCβ values were 2.15–1061.5 and 2.3–1135.9 μg kg^{-1}, respectively. All calibration curves showed excellent correlation ($r \geq 0.99$). The recovery of the analytes and internal standards (49.0–103.7 %) was reached with high precision (RSD <8 %). At different concentration levels, the variations in precision and accuracy, in terms of repeatability and in-laboratory reproducibility, were <11 %.

The new method is applicable to the routine analysis of commercial egg samples.

Keywords Veterinary drugs · Residue analysis · Eggs · Liquid chromatography-mass spectrometry · Validation

Introduction

Many classes of veterinary drugs, such as coccidiostats, lincosamides, macrolides, tetracyclines, sulfonamides, benzimidazoles, diterpenes, and diaminopyrimidines, are widely administered to laying hens for the treatment and prevention of diseases and as growth promoters (Stolker and Brinkman 2005). The inappropriate use of these drugs, or not respecting withdrawal periods, can result in the presence of residues (parent compound and metabolites) in eggs, increasing the potential risk to consumers due to allergic reactions in hypersensitive individuals and the induction of resistant strains of bacteria (World Health Organization 2011).

To control the residue problem, and protect the human consumers, several governmental authorities such as the European Union (EU), the Food and Drug Administration (FDA), and Codex Alimentarius established maximum residue levels (MRLs) for a variety of veterinary drugs in different foodstuffs (Commission Regulation 2010; Codex Alimentarius Commission 2012; Food and Drug Administration 2015). In eggs, the European Medicines Agency (EMEA) has set tolerances of 2 μg kg^{-1} for narasin, 50 μg kg^{-1} for lincomycin, 150 μg kg^{-1} for lasalocid and erythromycin, 200 μg kg^{-1} for tetracyclines and tylosin, 400 μg kg^{-1} for flubendazole, and 1000 μg kg^{-1} for tiamulin (Commission Regulation 2009; Commission Regulation 2010). The FDA has established MRLs for tetracyclines, erythromycin, and tylosin of 400, 25,

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and 200 μg kg^{-1}, respectively (Food and Drug Administration 2015). A Codex standard adopted an MRL of 400 μg kg^{-1} for tetracyclines, 50 μg kg^{-1} for erythromycin, and 300 μg kg^{-1} for tylosin at the 35th Session of the Codex Alimentarius Commission (Codex Alimentarius Commission 2012). According to EMEA, sulfonamides are prohibited for use in animals that produce eggs for human consumption and therefore no limit is set (Commission Regulation 2009).

A literature survey revealed several methods for the determination of veterinary drug residues in eggs, including liquid chromatography (LC) using ultraviolet (Christodoulou et al. 2007; de Paula et al. 2008), diode array (Furusawa 2003; Tolika 2011; Summa et al. 2015), and fluorescence detectors (Schneider and Donoghue 2003; Xie et al. 2011). However, these methods focused on one class of compounds, showing only moderate sensitivity and selectivity, and requiring long running times or complex extraction procedures. Recently, some published methods have used time-of-flight mass spectrometry (TOF-MS) to screen hundreds of compounds in eggs and other food matrices (Mol et al. 2008; Peters et al. 2009; Deng et al. 2011). However, methods based on TOF-MS are not considered in the Commission Decision 2002/657/EC regarding confirmatory methods (Commission Decision 2002).

LC coupled with tandem mass spectrometry (LC-MS/MS) provides higher selectivity and sensitivity than LC using conventional detectors. Furthermore, it fulfills the requirements demanded by the Commission Decision 2002/657/EC by combining analyte separation and structural information, providing unequivocal detection and confirmation of a substance and enabling the development of more reliable methods (Aerts et al. 1995; Kennedy et al. 1998).

Despite the advantages of LC-MS/MS, few analytical methods for the simultaneous analysis of multiclass drug residues in eggs have been described in the literature (Heller and Nochetto 2004; Heller et al. 2006; Jia et al. 2008; Mol et al. 2008; Frenich et al. 2010; Spisso et al. 2010; Jimenez et al. 2011; Capriotti et al. 2012; Kim et al. 2012; Nakajima et al. 2012; Robert et al. 2013; Dasenaki and Thomaidis 2015). This is probably due to differences in their chemical and physical properties, which make the extraction process and cleanup the most challenging steps. For these methods, sample preparation generally involves a solvent extraction step to promote the precipitation of proteins either alone (Mol et al. 2008; Spisso et al. 2010; Robert et al. 2013) or followed by an additional cleanup step using *n*-hexane (Dasenaki and Thomaidis 2015) or solid-phase extraction (SPE) (Jia et al. 2008; Frenich et al. 2010; Kim et al. 2012). The main advantage of the SPE procedure is the reduction in matrix effects and ion suppression. Nevertheless, additional cleanup steps involve time-consuming and laborious sample preparation protocols. Other extraction methodologies, including pressurized liquid extraction (PLE) (Jimenez et al. 2011) and quick, easy, cheap, effective, rugged, and safe (QuEChERS) (Capriotti et al. 2012; Nakajima et al. 2012), have

been evaluated. In other cases, the LC-MS method was not fully validated (Robert et al. 2013) or did not cover some pharmacological classes of primary interest in the egg, such as tetracyclines, macrolides, and/or coccidiostats (Capriotti et al. 2012; Nakajima et al. 2012; Spisso et al. 2010). For laboratories involved in official controls, it is important to have analytical methods available that are suitable for the simultaneous determination of several classes of veterinary drugs while optimizing the time and cost of analysis. To our knowledge, no validated methods have been published that use an extraction with acetonitrile without additional cleanup steps and are able to identify and simultaneously quantify coccidiostats, lincosamides, macrolides, tetracyclines, sulfonamides, benzimidazoles, diterpenes, and diaminopyrimidines in eggs by LC-MS/MS.

The objective of this study was to develop a rapid and sensitive LC-MS/MS method for the detection and confirmation of residues of 22 veterinary drugs and 8 different classes in eggs by using a simple and cheap extraction procedure. The whole procedure was validated according to the quality criteria of the Commission Decision 2002/657/EC and applied to the determination of residues in commercial egg samples.

Materials and Methods

Chemicals and Reagents

Standards of chlortetracycline (92 %), deuterated sulfamerazine (internal standard, 98 %), doxycycline (98.7 %), erythromycin (95.3 %), oxytetracycline (97.5 %), sulfadiazine (99.5 %), sulfadimethoxine (98 %), sulfamerazine (99.2 %), and sulfamethoxazole (99.5 %) were obtained from Dr. Ehrenstorfer (Bavaria, Germany). Cambendazole (internal standard, 99.8 %), demeclocycline hydrochloride (internal standard, 92.3 %), flubendazole (99.2 %), lasalocid A sodium salt (98 %), lincomycin hydrochloride (100.3 %), nigericin sodium salt (internal standard, ≥98 %), sulfacetamide (99.7 %), sulfachloropyridazine (99.4 %), sulfametazine (99.7 %), sulfamethoxypyridazine (99.8 %), sulfaquinoxaline (96 %), sulfathiazole (99.9 %), tetracycline hydrochloride (97.5 %), tylosin tartrate (87.9 %), and trimethoprim (99.5 %) were purchased from Sigma-Aldrich (MO, USA). Certified reference standards of narasin (86.1 %) and tiamulin (≥96.5 %) were available from the US Pharmacopeial Convention (MD, USA).

Acetonitrile and methanol (HPLC grade) were obtained from Panreac Química (Catalonia, Spain). Ammonium formate (≥97 %) was obtained from Acros Organics (NJ, USA). EDTA (≥98.5 %) and anhydrous sodium acetate (≥99 %) were purchased from Sigma-Aldrich (MO, USA). Formic acid (98–100 %) was obtained from Merck (MA, USA). Ultrapure water was obtained using a Milli-Q purification system from Millipore (MA, USA).

Standard Solutions

Individual stock standard solutions at 500 µg mL^{-1} were prepared in methanol for macrolides, sulfonamides, tetracyclines, lincomycin, narasin, nigericin, and trimethoprim, at 1 mg mL^{-1} in methanol for tiamulin and at 100 µg mL^{-1} in acetonitrile/methanol (50:50 v/v) for benzimidazoles. Lasalocid stock solution was provided as an acetonitrile solution at 100 µg mL^{-1} and used directly for further dilutions. All stock solutions were stored in amber bottles at 4 °C. Intermediate working solutions were prepared at the required concentrations by dilution of the standard stock solutions with methanol.

Blank Egg Samples

Blank egg samples, produced organically on a farm in Paraná (Brazil), were used for extraction development and validation studies. The fresh eggs were broken and homogenized, then 1 g was transferred into a 15-mL polypropylene centrifuge tube (TPP Techno Plastic Products, Trasadingen, Switzerland) and stored at −40 °C.

Sample Preparation

An aliquot of 50 µL of the internal standard (IS) solution (to obtain a final concentration of 150 µg kg^{-1} of nigericin, 200 µg kg^{-1} of deuterated sulfamerazine, 200 µg kg^{1} of demeclocycline, and 400 µg kg^{-1} of cambendazole) and 50 µL of methanol were added to 1 g of homogenized whole eggs. After mixing for 1 min and standing for 5 min, an aliquot of 400 µL of 100 mmol L^{-1} EDTA was added. The sample was vortexed for 1 min and allowed to rest for 5 min, then 5 mL of acetonitrile was added and the sample vortexed again for 1 min and sonicated for 10 min to homogenize the material with the solvent. After this, the sample was centrifuged (Eppendorf 5810-R, Hamburg, Germany) at 3220×g and 4 °C for 15 min. The supernatant was collected in a 15-mL polypropylene centrifuge tube and evaporated to 500 µL in a sample concentrator at 40 °C (CentriVap Labconco, MO, USA). The resultant extract was then diluted with 100 µL of methanol and 400 µL of water. The sample was vortexed for 1 min, sonicated for 10 min, and centrifuged at 3220×g and 4 °C for 15 min. Finally, 500 µL of supernatant was diluted with 500 µL of 5 mmol L^{-1} sodium acetate/acetonitrile/methanol (70:24:6 $v/v/v$). The sample was centrifuged at 20,817×g and 4 °C for 15 min and filtered through a PVDF syringe filter 0.22 µm pore size (Agilent HP, CA, USA) before injection.

LC-MS/MS Instrumentation and Conditions

LC-MS/MS analyses were performed using an Agilent 1200 HPLC System (Wilmington, USA) consisting of a G1312B binary pump, G1379B degasser, and G1316B column oven. The HPLC was connected to a CTC Sample Manager (Model 2777,

Waters Corporation, Milford, USA) operated at 20 °C. The HPLC system was coupled to an Applied Biosystems/MDS Sciex API 3200 Triple Quadrupole Mass Spectrometer (Toronto, Canada) equipped with a syringe pump (Harvard Apparatus, South Natick, USA) and an electrospray ionization (ESI) ion source. The ESI source was operated in positive ion mode. Analyte separation was achieved on an XBridge BEH C18 2.1 mm × 100 mm, 3.5 µm particle size (Waters Corporation, Milford, USA) column coupled with an XBridge BEH C18 2.1 mm × 10 mm, 3.5 µm particle size (Waters Corporation, Milford, USA) sentry guard cartridge maintained at 35 °C. The mobile phase comprised solvent A (water) and solvent B (acetonitrile/methanol 80:20 v/v), both containing 0.1 % formic acid. The gradient elution program used the following: 0–0.25 min, 5 % B; 0.25–0.50 min, increase to 25 % B; 0.50–6 min, maintained 25 % B; 6–8 min, linear increase to 85 % B; 8–9 min, increase to 100 % B; 9–14 min, maintained 100 % B; 14–15 min, decrease to 5 % B; and finally, 15–20 min, 5 % B. The flow rate was 400 µL min^{-1} and the injection volume 10 µL. The needle was washed with acetonitrile/methanol (50:50 v/v) containing 0.1 % formic acid between injections. Data acquisition was performed with MS Workstation by Analyst software version 1.4.2 (AB Sciex, MA, USA). Quantitation was performed in multiple reaction monitoring (MRM) mode. The ion transitions and the individual compound parameters, including the declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), and cell exit potential (CXP), are shown in Table 1. The ion-source parameters for ESI positive mode were as follows: curtain gas (CUR), 12 psi; collision gas (CAD), 10 psi; ion spray voltage (ISV), 5000 V; nebulizer gas (GS1), 45 psi; turbo gas (GS2), 50 psi; and temperature, 500 °C. The high-purity nitrogen and zero-grade air, used as the CUR, GS1, GS2, and CAD gases, were produced using a high-purity nitrogen generator from Peak Scientific Instruments (Chicago, USA).

Validation Procedure

The method was validated according to the requirements of the European Commission Decision 2002/657/EC (Commission Decision 2002) and Agência Nacional de Vigilância Sanitária do Brasil (Brasil 2012). The validation parameters included selectivity, limit of detection, limit of quantitation, decision limits, detection capability, linearity, carryover, matrix effect, recovery, precision, and stability.

Selectivity

Method selectivity was evaluated by analyzing 20 blank egg samples, obtained from two different sources, and checking the existence of interfering peaks in the analyte and IS retention time regions. The samples were prepared as described in the "Sample Preparation" section, but without the addition of the IS.

Table 1 Compound-dependent parameters and ion transitions of the analytes and ISs used for quantification

Compound	Precursor ion (m/z)	Product ion (m/z)	Dwell time (ms)	DP (V)	EP (V)	CEP (V)	CE (eV)	CXP (V)
Chlortetracycline	478.8	444.0	150	31	9.5	22	25	32
		462.0	150	31	9.5	22	23	8
Doxycycline	445.0	428.1	200	26	4.5	28	21	34
		154.1	200	51	8	22	45	4
Erythromycin	734.1	158.2	150	36	8.5	28	43	4
		116.2	150	36	8.5	28	59	4
Flubendazole	314.1	282.1	200	46	9.5	26	27	6
		123.1	200	46	9.5	26	47	4
Lasalocid	613.2	377.3	150	91	11	24	47	8
		595.5	150	91	11	24	31	42
Lincomycin	407.1	126.1	150	41	9	20	37	4
		70.1	150	41	9	20	89	4
Narasin	787.3	431.4	250	111	10.5	30	59	8
		531.4	250	111	10.5	30	51	4
Oxytetracycline	460.9	426.1	200	31	4.5	20	25	34
		201.1	200	31	4.5	20	51	4
Sulfacetamide	215.0	156.1	150	21	10.5	14	13	4
		92.1	150	21	10.5	14	29	4
Sulfachloropyridazine	284.9	156.0	150	26	9	16	19	4
		92.1	150	26	9	16	39	4
Sulfadiazine	251.1	156.1	150	31	9.5	16	19	4
		92.0	150	31	9.5	16	37	4
Sulfadimethoxin	311.0	156.1	250	41	10	16	25	4
		92.1	250	41	10	16	43	4
Sulfamerazine	265.0	156.1	250	36	10	16	21	4
		108.1	250	36	10	16	33	4
Sulfamethazine	279.0	186.2	150	46	10.5	16	21	4
		108.2	150	46	10.5	16	37	4
Sulfamethoxazole	253.9	156.0	150	31	9.5	16	19	4
		92.1	150	31	9.5	16	35	4
Sulfamethoxypyridazine	281.0	156.1	150	56	12	14	21	4
		92.2	150	56	12	14	41	4
Sulfaquinoxaline	301.0	156.0	200	31	10	16	21	4
		92.1	200	31	10	16	41	4
Sulfathiazole	255.9	156.1	250	46	11.5	16	19	4
		108.2	250	46	11.5	16	31	4
Tetracycline	445.0	410.0	250	36	9	20	23	8
		428.1	250	45	4.5	21	20	32
Tiamulin	494.1	192.2	150	41	10	24	27	4
		119.1	150	41	10	24	51	4
Trimethoprim	291.0	230.1	150	56	10	18	27	4
		123.1	150	56	10	18	31	4
Tylosin	916.1	174.2	200	71	11.5	32	51	4
		101.3	200	71	11.5	32	67	4
Cambendazole (IS)	303.0	217.1	150	41	9	22	39	4
		261.1	150	41	9	22	21	4
Demeclocycline (IS)	464.8	448.0	150	31	8.5	22	23	8
		430.0	150	31	8.5	22	27	8
Deuterated sulfamerazine (IS)	269.0	96.1	150	51	10.5	16	39	4
		160.0	150	51	10.5	16	23	4
Nigericin (IS)	747.3	703.5	150	126	12	32	45	58
		501.2	150	101	9	34	59	44

IS internal standard

Limit of Detection, Limit of Quantitation, Decision Limits, and Detection Capabilities

Limit of detection (LOD) and limit of quantitation (LOQ) were estimated from signal-to-noise ratios. To conduct this study, an aliquot of 50 μL of work standard solution and 50 μL of methanol was added to 1 g of blank egg sample. The samples were vortexed for 1 min, and a 400-μL aliquot of 100 mmol L^{-1} EDTA was added. The samples were subjected to the sample cleanup procedure described in the "Sample Preparation" section. The extract obtained was diluted with 100 μL of methanol and 400 μL of water to obtain a final concentration of 250 μg kg^{-1} for each analyte. The samples were vortexed again for 1 min, sonicated for 10 min, and

centrifuged at $3220 \times g$ and 4 °C for 15 min. Finally, the supernatant was diluted in series with 5 mmol L^{-1} sodium acetate/acetonitrile/methanol (70:24:6 *v/v/v*) and injected into the LC-MS/MS system until the smallest detectable peak was obtained. For each analyte, the LOD was calculated as the lowest concentration peak at a signal-to-noise ratio ≥3:1 and the LOQ was calculated as the lowest concentration peak at a signal-to-noise ratio ≥10:1.

Decision limits (CCα) were calculated using two different approaches, one for banned substances and one for MRL substances. For banned substances (doxycycline (DXC), sulfadiazine (SDZ), sulfadimethoxine (SDMX), sulfamerazine (SMR), sulfametazine (SMZ), sulfamethoxazole (SMX), sulfamethoxypyridazine (SMPZ), sulfacetamide (SCT), sulfachloropyridazine (SCP), sulfaquinoxaline (SQ), sulfathiazole (STZ), and trimethoprim (TMP)), the LOD was used as the CCα. For MRL substances (chlortetracycline (CTC), erythromycin (ERY), flubendazole (FLU), lasalocid (LAS), lincomycin (LIN), narasin (NAR), oxytetracycline (OTC), tetracycline (TC), tiamulin (TIA), and tylosin (TYL)), the CCα was calculated by analysis of 20 blank egg samples spiked with the analytes at MRL levels. The CCα was calculated as the concentration at the permitted limit plus 1.64 times the corresponding standard deviation.

For both substances, the CCβ was calculated by analysis of 20 blank egg samples spiked with the analytes at the CCα levels. The CCβ was calculated as the CCα plus 1.64 and multiplied by the corresponding standard deviation.

Linearity

The linearity of the method was demonstrated using blank matrix-matched calibration standards. An aliquot of 1 g of homogenized whole blank eggs was weighed in a polypropylene centrifuge tube (15 mL) and fortified with 50 μL of the appropriate work standard solution and 50 μL of the internal standard solution, to obtain final concentrations of 150 μg kg^{-1} of nigericin (NIG), 200 μg kg^{-1} of deuterated sulfamerazine (SMR-D4), 200 μg kg^{-1} of demeclocycline (DMC), and 400 μg kg^{-1} of cambendazole (CAM). The spiked samples were prepared as described in the "Sample Preparation" section.

The calibration curves were prepared at seven concentration levels plus a zero point (blank). For banned substances, the calibration curves were constructed at 0, 25, 37.5, 50, 62.5, 75, 87.5, and 100 μg kg^{-1} (equivalent to 0, 1, 1.5, 2, 2.5, 3, 3.5, and 4 lowest validation levels (LVL), respectively). For MRL substances, the calibration curves were constructed at 0, 1, 1.5, 2, 2.5, 3, 3.5, and 4 μg kg^{-1} for NAR; 0, 25, 37.5, 50, 62.5, 75, 87.5, and 100 μg kg^{-1} for LIN; 0, 75, 112.5, 150, 187.5, 225, 262.5, and 300 μg kg^{-1} for ERY and LAS; 0, 100, 150, 200, 250, 300, 350, and 400 μg kg^{-1} for CTC, OTC, TC, and TYL; 0, 200, 300, 400, 500, 600, 700, and 800 μg kg^{-1}

for FLU; and 0, 500, 750, 1000, 1250, 1500, 1750, and 2000 μg kg^{-1} for TIA (all equivalent to 0, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 MRL, respectively). For each compound, the calibration curve was used to confirm the linear relationship between analyte peak areas/IS peak areas and the analyte concentration/IS concentration. The slope, intercept, and regression coefficient (*r*) were calculated as regression parameters by weighted (1/x) linear. The linear correlation coefficient (*r*) was also required to be equal to or greater than 0.99.

CAM was used as internal standard for ERY, FLU, LIN, TIA, and TYL, while DMC was used as internal standard for tetracyclines. SMR-D4 was used as internal standard for sulfonamides and TMP, while NIG was used as internal standard for LAS and NAR.

Carryover Test

A carryover test was performed by injecting a triplicate of samples prepared at higher concentration levels (4 LVL and 2 MRL) alternating with the blank egg sample. No interfering peaks should have been observed in the blank egg chromatogram, with areas greater than 20 % of the peak areas at the lower concentration level of each analyte and 5 % of the IS peak area (Brasil 2012).

Matrix Effect

Matrix effect was evaluated at two different levels, 1 and 4 LVL for banned substances and 0.5 and 2 MRL for allowed substances.

The matrix effect analysis was performed using two different blank egg samples in sextuplicate spiked with all analytes and IS post-extraction and standard solution samples at the same concentration. The normalized effect of matrix (NEM) was calculated for each concentration level (analyte response in matrix/IS response in matrix vs. the analyte response in solution/IS response in solution). The relative standard deviation (RSD) of NEM had to be less than 15 % to indicate that the matrix effect was not significant (Brasil 2012).

Recovery (Extraction Efficiency)

Recovery was evaluated at three levels (1, 2, and 3 LVL for banned substances and 0.5, 1, and 1.5 MRL for allowed substances) by comparing the peak areas obtained from blank egg samples spiked with all analytes and IS before extraction (A) with those obtained from blank egg samples spiked with all analytes and IS post-extraction (B) (Brasil 2003). The percentage ratio of B/A was defined as recovery.

Precision and Trueness (Recovery)

The precision and trueness (recovery) of the methods were evaluated using measurements of repeatability and in-laboratory reproducibility (Commission Decision 2002). To perform this assay, the blank egg samples spiked with all analytes and IS were prepared at three levels (1, 2, and 3 LVL for banned substances and 0.5, 1, and 1.5 MRL for allowed substances). Repeatability was determined in 18 replicates at each level for three consecutive days and by the same analyst. In-laboratory reproducibility was evaluated in 18 replicates at each level, by a second analyst for three consecutive days.

The precision of the method was evaluated at each concentration level by calculating the relative standard deviation (RSD%) in repeatability and in-laboratory reproducibility conditions. The trueness (recovery) was evaluated at each concentration level by calculating the relative error (RE%), expressed as the difference between the mean of the experimental concentrations and the theoretical spiked concentrations divided by the theoretical spiked concentration.

Stability

Stability in Solution For stability studies of analytes and IS in solution, 50 mL of working solution was prepared with 5 mmol L^{-1} sodium acetate/acetonitrile/methanol (70:24:6 $v/v/v$) at a concentration of 50 ng mL^{-1} for banned substances and LIN; 2 ng mL^{-1} for NAR; 150 ng mL^{-1} for ERY and LAS; 200 ng mL^{-1} for CTC, OTC, TC, and TYL; 400 ng mL^{-1} for FLU; 1000 ng mL^{-1} for TIA; 150 ng mL^{-1} for NIG; 200 ng mL^{-1} for SMR–D4; 200 ng mL^{-1} for DMC; and 400 ng mL^{-1} for CAM. The working solution was fractionated into 40 aliquots (10 for each temperature). The aliquots were freshly evaluated and, after a period of 1, 2, and 3 weeks, stored at −40 and 4 °C (light-protected) and 20 °C (light-protected and light-exposed).

Stability was evaluated by comparing the peak areas obtained from freshly prepared working solution with those obtained after each period of storage. The results were expressed as the percentage of analyte remaining, considered stable for amounts ≥80 % (Croubels et al. 2003).

Stability in Matrix The stability of analytes in matrix was assessed by spiking 1 g of the blank egg sample ($n = 30$) with 50 μL of analyte solution, obtaining a concentration level of 2 LVL for banned substances and 1 MRL for allowed substances. The samples were vortexed for 1 min. One aliquot ($n = 6$) was prepared as described in the "Sample Preparation" section and analyzed immediately. The remaining aliquots were evaluated after periods of 1, 2, 3, and 4 weeks stored at −40 °C.

Stability was evaluated by comparing the mean recovery of analytes obtained from the freshly prepared sample with those obtained after each period of storage. The results were expressed as a percentage of analyte remaining. It is considered stable when the remaining amount is within the range of 80–110 % (Croubels et al. 2003).

Stability in Sample Post-Processing Stability of analytes in sample post-processing was assessed with a sample obtained as described in the "Stability in matrix" section.

The stability of the analytes and IS was evaluated by comparing the mean recovery obtained from fresh samples with those obtained after 24 and 48 h in the Sample Manager (20 °C, amber vials). The results were expressed as a percentage of analyte remaining, considered stable when the remaining amount is within the range of 80 to 110 % (Croubels et al. 2003).

Analysis of Real Samples

To evaluate the applicability of the validated method, 15 egg samples obtained from different local markets in Brazil were analyzed in triplicate. The samples were prepared as described in the "Sample Preparation" section.

Results and Discussion

LC-MS/MS Method Development

The mass spectrometer parameters were optimized by infusion experiments using individual working standard solutions of analytes and ISs prepared in acetonitrile/water (50:50 v/v) and containing different proportions of formic acid (0.05, 0.1, and 0.5 %) and ammonium formate (2, 3, and 5 mmol L^{-1}) as additives. For all compounds, the best signal intensity was observed when 0.1 % formic acid was used. Protonated ([M+H]$^+$) molecular ions were observed for the majority of analytes. However, for LAS, NAR, and NIG (IS), the sodium ([M+Na]$^+$) adduct ions were predominant.

Full-scan data acquisition was performed, and the analyte concentrations varied, to obtain an adequate signal intensity to optimize the individual compound parameters (DP, EP, CEP, CE, and CXP) by automatic MRM. When the precursor ions of DXC and TC were set (m/z 445), the same product ions were observed (m/z 428 and 410). According to Nakazawa et al. (1999) and De Ruyck and De Ridder (2007), the product ions at m/z 428 corresponded to [M+H–NH$_3$]$^+$ and at m/z 410 to [M+H–H$_2$O–NH$_3$]$^+$ for TC and [M+H–NH$_3$–H$_2$O]$^+$ for DXC. However, the relative abundance of these product ions differed significantly; m/z 428 was the most intense for DXC and m/z 410 for TC. For sulfonamides, the common product ions were obtained (m/z 92, 108, and 156), indicating a well-

defined fragmentation profile (Gehring et al. 1996). At the conclusion of MS/MS optimization, the two most intense fragment signals for each compound were obtained. The most intense was used for quantification and the second most intense for peak qualification (see Table 1).

Optimization of the source parameters (CUR, CAD, ISV, GS1, GS2, and temperature) was accomplished through flow injection analysis (FIA) by coupling the chromatograph with the mass spectrometer. The pump was operated at 200 µL min^{-1} using an isocratic system with a mobile phase of acetonitrile/water (50:50 v/v) containing 0.1 % formic acid.

Due to the distinct physicochemical characteristics of the substances, an XBridge BEH C18 column (100 mm × 2.1 mm, 3.5 µm particle size) coupled with an XBridge BEH C18 precolumn (10 mm × 2.1 mm, 3.5 µm particle size) was used during method development. For mobile phase composition, several combinations of methanol, water, and acetonitrile were tested, maintaining 0.1 % formic acid as the additive under all conditions. Flow rate (200–400 µL min^{-1}) and column oven temperature (30–40 °C) were also varied to achieve optimum run times and peak shapes.

Various gradient programs were tested, taking the distinct physical–chemical characteristics of the compounds into consideration. In order to retain the most polar substances, an initial gradient with 95 % water was established. For all conditions tested, the acetonitrile/methanol mixture provided better ionization of analytes than acetonitrile when employed as an organic modifier, especially for the sodium adducts of NAR, LAS, and NIG (IS). Furthermore, complete separation of tetracycline and doxycycline isomers (with mass transitions in common; see Table 1) was achieved, indicating the excellent specificity of the developed method.

At the end of method development, the best overall sensitivity and peak shape were achieved by maintaining the column at 35 °C with a gradient mobile phase consisting of (A) water and (B) acetonitrile/methanol (80:20, v/v), both containing 0.1 % formic acid. The optimum run time was achieved by maintaining the flow rate at 400 µL min^{-1}. Representative MRM chromatograms of the LC-MS/MS method are presented in Fig. 1.

Sample Preparation Optimization

The simultaneous determination of 22 veterinary drug residues belonging to eight classes was a challenging task due to their very different chemical structures and properties. Therefore, appropriate sample treatment was essential for obtaining reliable results.

In order to remove lipids and proteins and achieve the best recovery of analytes and ISs, different extraction procedures were tested, including protein precipitation (PP) and SPE. All extraction procedures were performed by preparing a blank egg sample, spiked with analytes and ISs at 250 µg kg^{-1}, in six replicates.

The most frequently organic solvents used as extracting agents are methanol and acetonitrile because they both allow protein precipitation as the analyte extraction. Many authors prefer acetonitrile (Mol et al. 2008; Dubreil-Cheneau et al. 2009; Peters et al. 2009; Frenich et al. 2010; Spisso et al. 2010; Robert et al. 2013) over methanol, because methanol extracts many matrix compounds, complicating further cleanup procedures and making difficult the sample analysis. Acetonitrile is commonly employed in sample preparation as it provides protein precipitation with high extraction efficiency and minimum co-extraction of lipids.

Different volumes of acetonitrile (2.5, 5, and 10 mL) were evaluated for 1 g of egg matrix. Using 5 mL of acetonitrile as a precipitating agent caused rapid protein precipitation, forming a dense precipitate that was easily removed by centrifugation. In addition, it demonstrated the highest efficiency for compound extraction (>50 %) with adequate reproducibility (RSD <8 %). During the experiments, it was observed that there was a slight improvement of the recoveries when increasing the volume of the acetonitrile, but not significant to justify the use of higher amount of solvent.

Tetracyclines can form complexes with metal ions (e.g., Ca^{2+} and Mg^{2+}) present in the sample. Thus, an aliquot of 400 µL of 100 mmol L^{-1} EDTA was added to the sample as a competing chelating agent. This led to improved recoveries (>60 %) for CTC, OTC, and TC.

To improve extract cleanup, SPE purifications were performed with buffers at different pH values (pH 3–7) with Oasis HLB cartridges, recommended for polar and non-polar compounds. Briefly, the extract obtained from the precipitation of proteins with acetonitrile was evaporated until dryness and redissolved with the extraction buffer, which consisted of the following: pH 3 citric acid buffer, pH 4.5 acetic acid buffer, pH 6 acetic acid buffer, and pH 7 phosphate buffer. At pH 3 and 4.5, ERY recovery was <30 % as this analyte was unstable under acidic conditions. At pH 6 and 7, recoveries were higher than 60 % for most sulfonamides, but <40 % for DXC, TC, and DMC (IS). Among the pH evaluated, low recoveries and inadequate repeatability (RSD >20 %) were observed for the majority of analytes. According to Peters et al. (2009), egg proteins are smaller and water-soluble, interacting strongly with the cartridge sorbent and consequently interfering with analyte adsorption.

Finally, PP with acetonitrile was selected for sample preparation. When compared with SPE, this procedure presents more advantages as lower cost of analysis, smaller amount of residual chemicals generated, and less time consuming for sample preparation.

Considering the previous report (Dubreil-Cheneau et al. 2009; Spisso et al. 2010), a mixture of 5 mmol L^{-1} sodium acetate/acetonitrile/methanol (70:24:6 $v/v/v$) was used as the
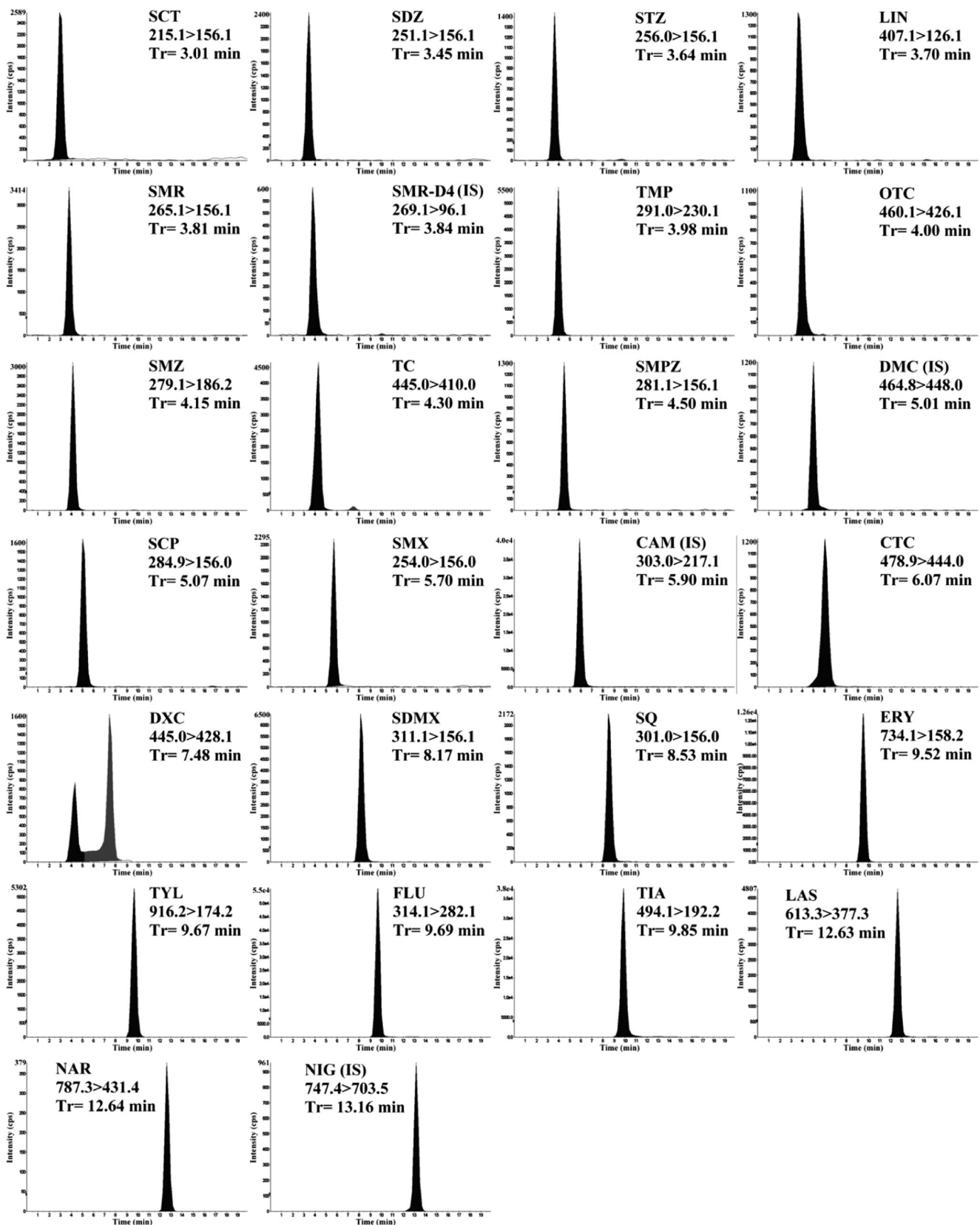
Fig. 1 MRM chromatograms of egg sample spiked with analytes and internal standards at concentrations of 4 LVL and 2 MRL
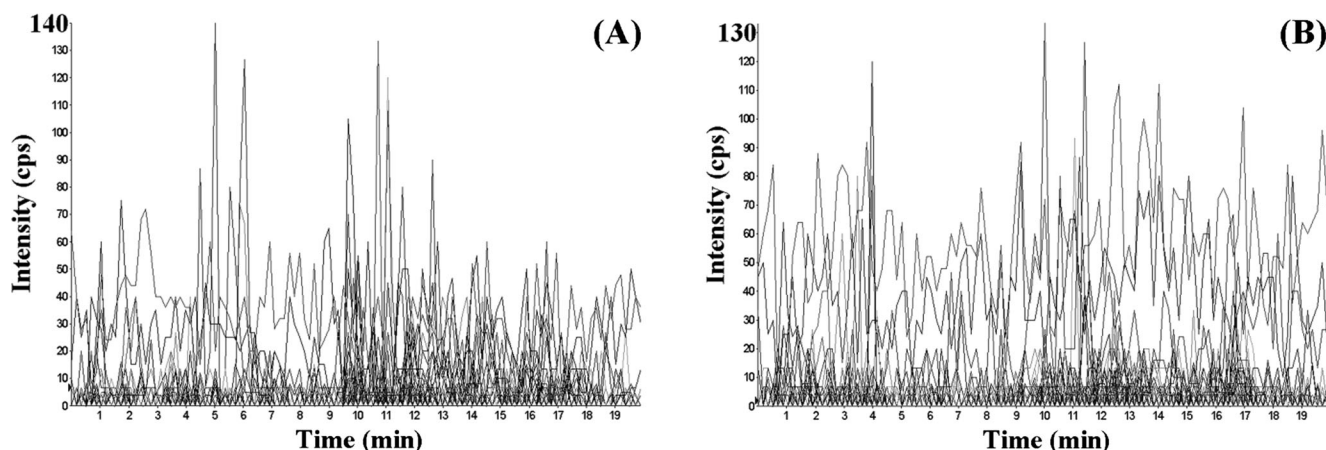
Fig. 2 Chromatograms obtained by LC–MS/MS for the selectivity study: **a** blank egg sample A and **b** blank egg sample B

solvent for reconstitution of the extract to ensure the stability of sodium adducts.

Method Validation

Selectivity

The chromatograms obtained using different blank egg samples are presented in Fig. 2. The results showed no interfering peaks co-eluted with analytes and ISs. The developed method was, therefore, considered to be selective.

Limit of Detection, Limit of Quantitation, Decision Limits, and Detection Capabilities

As demonstrated in Table 2, the developed method demonstrated high sensitivity with low LOD (0.37–7.5 µg kg^{-1}) and LOQ (1.25–20.0 µg kg^{-1}). In general, these results are similar or better than previously published methods.

For banned substances, the CCα and CCβ values ranged from 0.62 to 7.5 and 0.65 to 8.1 µg kg^{-1}, respectively. For MRL substances, the CCα and CCβ values ranged from 2.15 to 1061.5 and 2.3 to 1135.9 µg kg^{-1}, respectively. When compared to the method proposed by Spisso et al. (2010), the CCα and CCβ values for ERY, LAS, LIN, NAR, and TYL were considerably lower (2.3 to 262.7 µg kg^{-1}). For CTC, DXC, LAS, OTC, and SMX, the CCα and CCβ values were lower than those in Capriotti et al. (2012), which were in the range of 13.3 to 283 µg kg^{-1}. The methods published by Heller and Nochetto (2004), Heller et al. (2006), Jia et al. (2008), Mol et al. (2008), Deng et al. (2011), Kim et al. (2012), Nakajima et al. (2012), and Dasenaki and Thomaidis (2015) did not report the values of CCα and CCβ. Considering the importance of performance characteristics required by the Commission Decision 2002/657/EC, the lack of evaluation

of CCα and CCβ may compromise the confidence and suitability of the method to the intended use.

Linearity

The calibration curves obtained were linear with correlation coefficients (r) higher than 0.993 for all analytes in all

Table 2 Limits of detection (LOD), limits of quantification (LOQ), decision limits (CCα), and detection capabilities (CCβ) for target analytes in the developed method

Analyte	MRL (µg kg^{-1})	LOD	LOQ	CCα	CCβ
CTC	200	2.5	12.5	214.5	227.3
DXC	–	7.5	20.0	7.5	8.1
ERY	150	2.5	10.0	156.5	169.6
FLU	400	1.0	5.0	414.4	443.4
LAS	150	2.5	10.0	159.0	169.0
LIN	50	1.25	3.12	53.8	57.0
NAR	2	0.25	0.5	2.15	2.3
OTC	200	7.5	20.0	211.8	227.6
SCT	–	7.5	20.0	7.5	8.1
SCP	–	2.5	10.0	2.5	2.6
SDZ	–	0.62	2.5	0.62	0.67
SDMX	–	0.62	2.5	0.62	0.65
SMR	–	1.25	5.0	1.25	1.3
SMZ	–	0.62	2.5	0.62	0.66
SMX	–	2.5	10.0	2.5	2.6
SMPZ	–	7.5	20.0	7.5	8.1
SQ	–	2.5	10.0	2.5	2.7
STZ	–	7.5	20.0	7.5	8.0
TC	200	2.5	12.5	212.6	225.5
TIA	1000	0.37	1.25	1061.5	1135.9
TMP	–	0.62	2.5	0.62	0.68
TYL	200	0.75	2.5	212.2	216.1

Table 3 Variation of the normalized effect of matrix (NEM) of analytes in the study, calculated for assessing matrix effects ($n = 6$)

Analyte	Nominal concentration (µg kg^{-1})	Blank egg sample A			Blank egg sample B		
		NEM	Mean ± SD	NEM (RSD%)	NEM	Mean ± SD	NEM (RSD%)
CTC	100	0.9	1.0 ± 0.1	14.1	0.9	0.9 ± 0.01	1.5
	400	1.1			0.9		
DXC	25	1.1	1.0 ± 0.1	9.5	0.9	1.0 ± 0.13	13.5
	100	1.0			1.0		
ERY	75	1.1	1.2 ± 0.1	11.9	1.0	1.1 ± 0.11	10.1
	300	1.3			1.2		
FLU	200	0.4	0.5 ± 0.06	12.1	0.6	0.5 ± 0.03	5.5
	800	0.5			0.5		
LAS	75	1.1	1.2 ± 0.2	13.9	1.3	1.3 ± 0.06	5.0
	300	1.3			1.2		
LIN	25	0.8	0.9 ± 0.1	14.4	0.9	1.0 ± 0.1	10.1
	100	1.0			1.1		
NAR	1	1.4	1.3 ± 0.1	8.4	9.2	8.6 ± 1.0	11.2
	4	1.2			7.9		
OTC	100	1.0	1.0 ± 0.05	5.0	0.8	0.9 ± 0.11	12.3
	400	1.1			1.0		
SCT	25	0.6	0.6 ± 0.03	5.4	0.6	0.6 ± 0.02	2.7
	100	0.6			0.6		
SCP	25	0.7	0.8 ± 0.08	9.9	0.9	0.9 ± 0.02	2.5
	100	0.8			0.9		
SDZ	25	0.9	1.0 ± 0.08	8.7	1.1	1.1 ± 0.05	4.1
	100	1.0			1.1		
SDMX	25	0.9	0.9 ± 0.04	5.1	0.8	0.8 ± 0.03	3.8
	100	0.8			0.8		
SMR	25	0.9	0.9 ± 0.01	1.5	1.0	1.0 ± 0.05	5.2
	100	0.9			0.9		
SMZ	25	1.1	1.1 ± 0.05	4.6	1.1	1.1 ± 0.08	9.4
	100	1.0			1.0		
SMX	25	0.7	0.8 ± 0.07	8.3	0.9	0.9 ± 0.02	2.5
	100	0.8			0.9		
SMPZ	25	0.7	0.8 ± 0.09	11.5	0.6	0.6 ± 0.05	7.5
	100	0.9			0.6		
SQ	25	0.8	0.8 ± 0.03	3.9	0.7	0.8 ± 0.03	4.6
	100	0.8			0.8		
STZ	25	0.6	0.7 ± 0.1	13.8	0.8	0.9 ± 0.09	10.3
	100	0.8			0.9		
TC	100	0.9	1.0 ± 0.1	14.1	0.9	1.0 ± 0.06	6.2
	400	1.1			1.0		
TIA	500	0.4	0.4 ± 0.02	5.7	0.7	0.6 ± 0.03	4.3
	2000	0.4			0.6		
TMP	25	0.8	0.9 ± 0.04	4.9	0.7	0.7 ± 0.03	4.0
	100	0.9			0.8		
TYL	100	0.7	0.7 ± 0.04	5.3	1.2	1.3 ± 0.2	14.4
	400	0.7			1.4		

concentration ranges. Additionally, at all concentrations, the results exhibited variations in precision and accuracy <15 %, guaranteeing a reliable response, regardless of the concentration.

Table 4 Precision and accuracy of analytes obtained in egg samples by LC-MS/MS ($n = 18$)

Analytes	Level (µg kg^{-1})	Repeatability		Within-laboratory reproducibility	
		Precision (RSD%)	Accuracy (RE%)	Precision (RSD%)	Accuracy (RE%)
CTC	100	3.5	−0.9	5.5	2.1
	200	2.8	−0.6	3.8	0.5
	300	1.0	0.3	6.3	−1.7
DXC	25	4.4	−3.6	5.6	−4.8
	50	5.7	−2.0	4.7	−1.4
	75	1.1	−1.5	1.6	2.5
ERY	75	5.9	−2.7	6.4	2.7
	150	2.2	6.4	5.2	5.8
	225	6.4	2.2	3.8	0.2
FLU	200	5.1	−4.4	3.5	−4.4
	400	3.8	−2.6	1.2	0.5
	600	2.8	0.2	4.1	5.1
LAS	75	9.5	−6.8	7.5	−9.5
	150	7.4	−5.8	3.5	−7.1
	225	6.7	2.5	4.1	5.2
LIN	25	2.1	−4.4	0.8	−3.6
	50	4.9	2.9	5.8	0.8
	75	4.7	−1.3	5.4	3.6
NAR	1	5.5	1.0	6.5	−9.1
	2	5.0	2.7	4.0	2.6
	3	2.5	0.5	1.9	−1.5
OTC	100	2.6	−1.7	4.4	1.3
	200	1.0	0.4	3.1	−2.2
	300	2.1	−0.1	3.6	2.1
SCT	25	8.9	2.4	6.1	8.6
	50	7.6	1.2	3.2	9.0
	75	4.5	−0.9	2.3	6.0
SCP	25	2.7	−4.5	7.1	0.8
	50	2.7	3.2	4.7	2.9
	75	3.4	0.4	1.9	3.4
SDZ	25	7.3	−2.4	7.2	0.4
	50	3.9	2.1	8.9	2.4
	75	1.4	−0.3	3.3	6.2
SDMX	25	5.0	0.1	4.5	0.9
	50	2.6	−0.08	4.0	5.1
	75	2.8	2.2	3.4	3.6
SMR	25	2.2	−1.2	10.6	3.3
	50	7.1	−4.4	7.3	5.9
	75	1.7	−1.0	6.8	−1.6
SMZ	25	2.7	−1.7	3.0	2.4
	50	1.6	−0.2	3.2	5.8
	75	2.4	1.1	4.5	0.2
SMX	25	2.6	−4.8	1.3	−1.3
	50	3.4	2.7	0.9	−1.8
	75	4.2	−1.2	5.1	1.3
SMPZ	25	9.7	1.1	10.6	4.0
	50	6.7	2.0	9.3	4.1
	75	5.7	3.9	7.9	5.9
SQ	25	1.2	−4.2	7.0	−2.9
	50	1.6	0.3	4.3	0.7
	75	3.0	1.2	3.7	6.0
STZ	25	4.5	−2.2	8.3	4.0
	50	7.3	−0.6	9.0	−2.2
	75	5.6	0.04	7.0	1.8
TC	100	3.2	−0.8	3.9	−1.5
	200	3.3	1.5	2.7	1.2
	300	1.7	1.4	2.2	4.4
TIA	500	2.4	0.1	3.1	−2.7
	1000	0.9	−2.3	7.3	0.7
	1500	5.4	1.7	7.1	1.3
TMP	25	4.1	−4.4	8.8	6.4
	50	6.5	−0.9	8.1	3.8
	75	3.5	−1.4	7.8	0.05
TYL	100	1.9	−1.8	4.8	−0.1
	200	2.6	−0.07	2.1	0.6
	300	2.6	−1.8	4.2	0.6
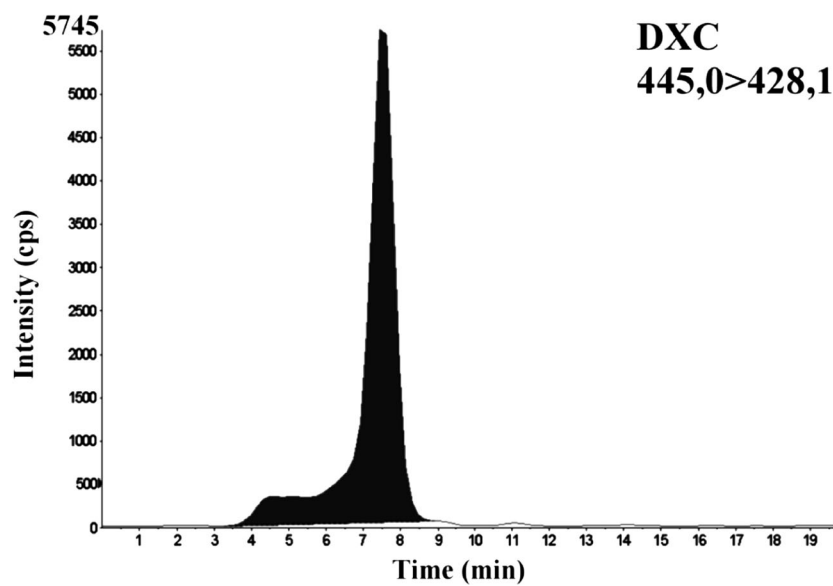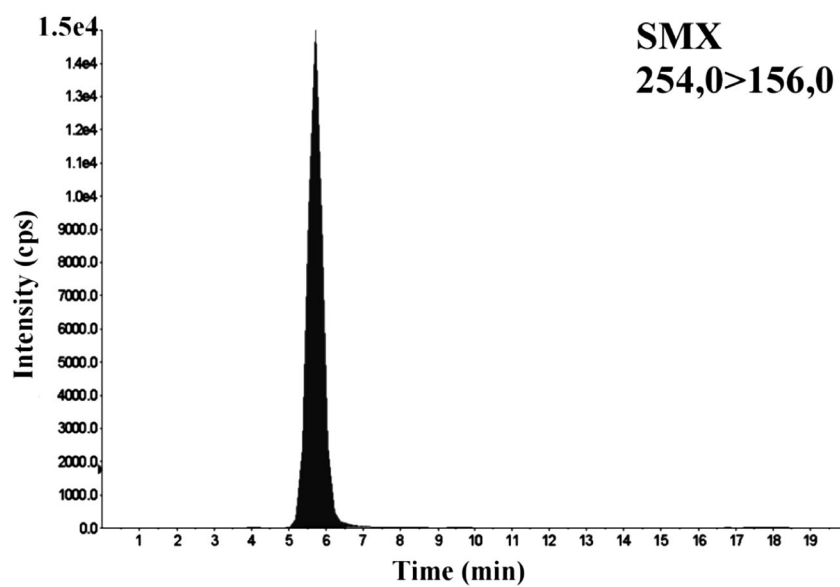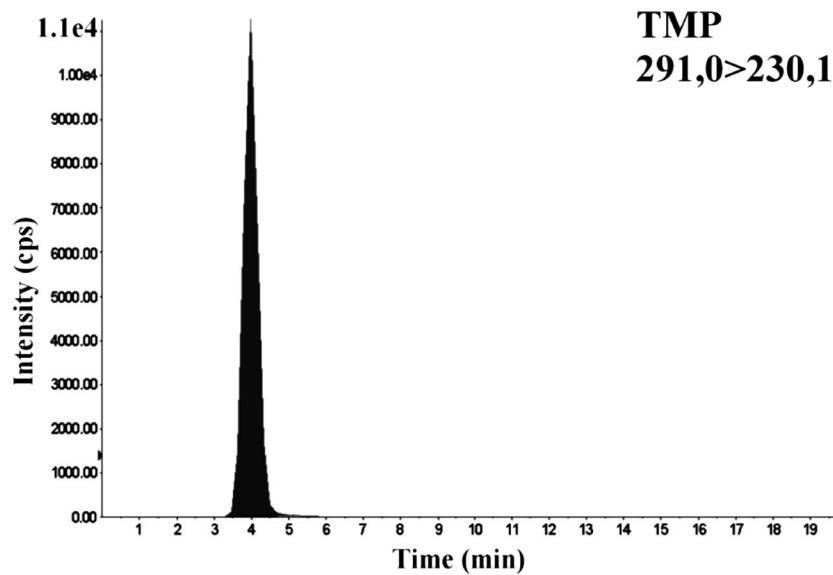
◀ **Fig. 3** MRM chromatograms of an egg sample with residues of trimethoprim (TMP) at 241.1 µg kg^{-1}, sulfamethoxazole (SMX) at 635.2 µg kg^{-1}, and doxycycline (DXC) at 538.0 µg kg^{-1}

Carryover

No significant interfering peaks (<1 % of the peak areas) were observed at the retention times of the analytes and ISs between injections. Thus, there was no risk of carryover contamination during sample analysis.

Matrix Effects

The variations of the NEM for each compound were <15 %, indicating that the effects of the biological matrix on analyte response was insignificant (Table 3).

Recovery (Extraction Efficiency)

For all compounds, satisfactory recoveries (49.0–103.7 %) were achieved, with high reproducibility (RSD <8 %). Recovery values were >80 % for more than 70 % of the compounds. Protein precipitation with acetonitrile was therefore considered effective for extracting veterinary drugs from hen eggs.

Precision and Trueness (Recovery)

The results of precision and trueness (recovery) are shown in Table 4. The new method was precise for all analytes, with RSD varying from 0.9 to 9.7 % for repeatability and from 0.8 to 10.6 % for in-laboratory reproducibility analysis. These results were in accordance with the "between one half and two thirds" value recommended in the Commission Decision 2002/657/EC for each concentration level.

The method also showed satisfactory trueness (recovery), with RE ranging from −6.8 to +6.4 % for repeatability and from −9.5 to +9.0 % for in-laboratory reproducibility analysis. These results were in accordance with the Commission Decision 2002/657/EC that recommended recoveries, in terms of bias, between −30 % and +10 % for analytes with concentrations of 1–10 µg kg^{-1} and between −20 % and +10 % for analytes with concentrations >10 µg kg^{-1}.

Stability

Stability in Solution A threshold of 80 % was applied for stability in solution, considering the high intrinsic variability of the MS detector (Croubels et al. 2003). After 1 week, degradation of DMC, DXC, LAS, NAR, SMX, SMZ, TC, TIA, and TMP was observed under all storage conditions. Fast photodegradation was observed in tetracyclines; similar observations have previously been reported (Oka et al. 1989;

Soeborg et al. 2004). Only SDMX was stable under all storage conditions after 3 weeks. Thus, it was concluded that working solutions should be freshly prepared before analysis.

Stability in Matrix

For stability in matrix, a threshold of 80–110 % was applied, considering the minimum trueness of quantitative methods that establish an accuracy of −20 to +10 % for analytes with a concentration >10 µg kg^{-1}.

It is known that the pH of the yolk and egg white are different, 6 and 7.6, respectively (Kan and Petz 2000). However, the egg white pH increases during the storage period and can reach up to 9.5 (Pereira et al. 2014). According to Soeborg et al. (2004), this alkaline pH contributes to the degradation of chlortetracycline (*iso*-chlortetracycline formation) and to its maximum complexation with Ca^{2+} ions. This could explain the low stability of tetracyclines, which was less than 2 weeks. Given the structural similarity of the tetracyclines, it is expected that both of them undergo the same type of degradation pattern.

Degradation of SMPZ and STZ was observed after 1 week of storage. After 2 weeks, degradation of SCT, SDZ, SMZ, SQ, LAS, TYL, TMP, and TIA was observed. SMX and ERY were stable for less than 3 weeks and SMR, SDMX, LIN, and FLU for less than 4 weeks. Only NAR and SCP remained stable after 4 weeks, keeping a threshold of 81 and 86 %, respectively. Therefore, egg samples fractionated should not be stored longer than 1 week before analysis.

Stability in Sample Post-Processing No significant differences were demonstrated after 48 h in Sample Manager (20 °C). Thus, excellent stability was shown for all compounds.

Sample Analyses

The newly developed and validated HPLC-MS/MS method was applied to 15 egg samples acquired from a local market. Figure 3 shows the MRM chromatogram of one sample containing residues of doxycycline (538.0 µg kg^{-1}), sulfamethoxazole (635.2 µg kg^{-1}), and trimethoprim (241.1 µg kg^{-1}), the use of which is banned in animals that produce eggs for human consumption. This result shows the importance and potential application of the new method.

Conclusion

A new, reproducible, sensitive, fast, and fully validated LC-MS/MS method was developed for the simultaneous

determination of 22 veterinary drugs in eggs. Data from the validation study demonstrated that the method was selective, linear, precise, accurate, and free of residual and matrix effects. The newly developed method was successfully applied to real samples and found to be applicable to routine analysis, enabling sample compliance assessment according to regulatory limits established in the EU regulations.

Compliance with Ethical Standards

Conflict of Interest Flávia Lada Degaut Pontes declares that she has no conflict of interest. João Cleverson Gasparetto declares that he has no conflict of interest. Thais Martins Guimarães de Francisco declares that she has no conflict of interest. Heloise Calzado Goetzke declares that she has no conflict of interest. Letícia Paula Leonart declares that she has no conflict of interest. Daniel Altino de Jesus declares that he has no conflict of interest. Roberto Pontarolo declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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