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Development of Radiometric and Allied Analytical Methods and Strategies to Strengthen National Residue Control Programmes for Antibiotic and Anthelmintic Veterinary Drug Residues

Final Report of a Coordinated Research Project

IAEA
International Atomic Energy Agency

DEVELOPMENT OF RADIOMETRIC AND ALLIED ANALYTICAL METHODS AND STRATEGIES TO STRENGTHEN NATIONAL RESIDUE CONTROL PROGRAMMES FOR ANTIBIOTIC AND ANTHELMINTIC VETERINARY DRUG RESIDUES

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FINAL REPORT OF A COORDINATED RESEARCH PROJECT

PREPARED BY THE
JOINT FAO/IAEA DIVISION OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE

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For further information on this publication, please contact:

Food and Environmental Protection Section
International Atomic Energy Agency
Vienna International Centre
PO Box 100
1400 Vienna, Austria
Email: Official.Mail@iaea.org

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FOREWORD

Awareness of food safety is rising among consumers, and many importing countries implement food control regulations to guarantee the quality and safety of imported foods for their consumers. Many developing countries have also taken steps to put in place control systems that encourage responsible use of veterinary medicines to combat possible drug resistance, control drug residues and ensure compliance with international and national standards. However, these countries still require the necessary know-how and skills to protect local consumers and to access international markets.

One significant constraint is the capacity of laboratory services to generate surveillance data using reliable and cost effective analytical methods validated to national and international standards.

The IAEA coordinated research project (CRP) on the Development of Radiometric and Allied Analytical Methods to Strengthen National Residue Control Programmes for Antibiotic and Anthelmintic Veterinary Drug Residues was initiated in 2009 to conduct work on robust nuclear and related technologies suitable for the screening and confirmatory analysis of residues of veterinary medicines, including antimicrobials and anthelmintics commonly used in animal production, with public health and trade significance. The CRP also explored mechanisms to enhance networking among research institutions involved in research on pharmacologically active veterinary drug residues in food (primarily) and environmental samples.

The project was implemented by the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture between 2009 and 2014 and involved eleven research contracts and one technical contract, five research agreements and one institution. The CRP was a continuation of the CRP on the Development of Strategies for the Effective Monitoring of Veterinary Drug Residues in Livestock and Livestock Products in Developing Countries and the key findings are also summarized in this publication.

The IAEA officers responsible for this publication were A. Cannavan and J.J. Sasanya of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture.

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SUMMARY

1. BACKGROUND

This technical document (TECDOC) is an output of a Joint FAO/IAEA CRP "Development of Radiometric and Allied Analytical Methods and Strategies to Strengthen Residue Control Programs for Antibiotic and Anthelmintic Veterinary Drug Residues" coded as D52036, conducted between 2009 and 2014 to support monitoring selected veterinary antibiotic and anthelmintic drug residues in order to safeguard consumer safety. It also includes a summary of work done under the predecessor CRP D32022 completed in December 2006.

Rapid growth in the livestock sector in many developing Member States has resulted in changing production practices and increase in international trade for food products of animal origin. However, disease burdens as well as the corresponding prophylactic or therapeutic use of veterinary antimicrobials and anthelmintics have also increased. Although many countries encourage responsible use of these medicines, there are significant constraints, including availability of suitable analytical methods to monitor and control resultant residues.

Approaches for the detection of veterinary drug residues include microbiological, immunochemical, chromatographic and spectrometric techniques that involve use of stable isotopes, among others. While these may fulfil suitability criteria, such as method sensitivity, they require multiple, time-consuming steps for extraction, sample clean-up or pre-concentration prior to analysis and often lack the robustness necessary for successful application in developing Member States. These issues can be addressed by developing methods utilizing nuclear, isotopic and related technologies. This is the goal of the D52036, building on its predecessor D32022, as part of an IAEA project entitled Integrated Control of Food and Environmental Hazards.

The project D52036 also aimed at establishing a harmonised network of expertise able to share knowledge and transfer technology to strengthen national residue control programs of Member States to improve food safety, help in potential combating of drug resistance and the compliance with harmonized Codex Alimentarius standards. The specific objectives were to develop and validate quality assured screening methods for the detection of antimicrobial and anti–parasitic drugs based on the novel radioimmunoassay (RIA) technology developed under D32022, using radioactive labelled streptavidin in a competitive immunoassay format and confirmatory methods using isotope-dilution mass spectrometric techniques with a particular focus on rapid sample preparation.

2. OBJECTIVE

The information reported in this TECDOC brings together the results of the work completed under the CRP D52036 with a summary of the key results from CRP D32022. The information is useful for research on, and technology transfer to facilitate, veterinary drug and associated residue testing in food and environmental samples. The TECDOC presents a source of standard operating standards that can be used for step-by-step laboratory procedures.

3. SCOPE

This TECDOC consists of research papers on radiometric and allied analytical techniques including LC with associated detectors such as mass spectrometry, antibody based methods, RIA and application of radioisotopes. Research work addressed questions raised by over 17 institutions on analysis of residues of various veterinary drugs in mostly food but also environmental samples such as water, soil and pasture plants.

Competitive immunoassays and other screening techniques, including High Performance Thin Layer Chromatography (HPTLC) with optical scanning and/or autoradiography; whole body autoradiography and liquid scintillation counting involving ^{14}C–flumequine used to study the pharmacokinetics of flumequine in fish are reported.

Emphasis was placed in D52036 on anti–parasitic drugs widely used in developing countries, such as benzimidazoles and macrocyclic lactones, and compounds highlighted by the Joint FAO/WHO/OIE Experts Meeting on Critically Important Antimicrobials, including widely used substances such as aminoglycosides, cephalosporins, macrolides, quinolones, sulphonamides and tetracyclines among others.

4. STRUCTURE

The report begins with work on isotopes dilution LC–MS/MS multianalyte methods, a study on the pharmacokinetics of flumequine in farmed sea bream, followed by confirmatory method multiple aminoglycosides as well as ELISA based investigation of enrofloxacin and ciprofloxacin in poultry and chicken meat. The report then addresses the investigation of CAP and its residues in food and environmental samples in Mongolia and the application of an isotope dilution method to investigate natural occurrence of CAP in poultry litter.

The middle part of the report addresses both enzyme and radio based immunoassay techniques for a number of drug residues. This is followed by screening methods for antihelmintics in various products, chromatographic methods for sulphonamide antibacterial residues and finally prohibited drugs such as nitrofurans and their metabolites in animal products including shrimps.

5. BRIEF REVIEW AND ANALYSIS OF RESEARCH PAPERS

This section summarizes the papers reported in the TECDOC and the conclusions of CRP D52036.

Paper 1: Investigations of multi–analyte screening and confirmatory analytical methods (based on use of stable isotopes) were successfully conducted by the Center for Food and Drug Analysis, Busan Regional Food and Drug Administration, Ministry of Food and Drug Safety as well as the Animal, Plant and Fisheries Quarantine and Inspection Agency. The methods were also applied for analysis of residues of tetracyclines, sulphonamides, penicillins, cephalosporins, macrolides, amphenicols, quinolones and aminoglycosides in animal products and the environment. The phenomenon of cross talk was also investigated.

Paper 2: The pharmacokinetic profile of flumequin (FLU) in sea bream was also studied involving the use of whole body autoradiography and liquid scintillation counting and ^{14}C–

labelled FLU. The study demonstrated that FLU is suitable for treating diseases in Tunisian/Mediterranean sea bream.

Paper 3: A robust quantitative and confirmatory method was also developed for simultaneous determination of 16 aminoglycoside residues in porcine tissues (muscle, liver and kidney) using liquid chromatography tandem mass spectrometry (LC–MS/MS). A combination of solid phase extraction columns conditioned at varying pH levels was used in sample preparation.

Paper 4: A multi–parametric enzyme linked immunosorbent assay (ELISA) technique was investigated for detection of enrofloxacin and ciprofloxacin residues in chicken. Work also involved the production of relevant antibodies. A corresponding confirmatory LC–MS/MS method was also developed and validated for effective use in chicken plasma, muscle, fat/skin, kidney and liver samples. Broiler chickens were also used to study depletion of enrofloxacin.

Paper 5: Screening (ELISA and HPLC) and confirmatory (LC–MS/MS) techniques for investigation of naturally occurring Chloramphenicol (CAP) residues in plant, animal and environmental samples were studied. Examples of the plants included Artemisia and Thalictrum. The study found that Mongolian pastures do not always contain high concentrations of CAP and that no single herb is associated with the residues in animal products.

Paper 6: A sensitive and specific LC–MS/MS isotope dilution method was used to investigate trace levels of CAP in the experimental samples, attributed to *S. venezuelae* from the environment. *S. venezuelae* was found not to be viable after about 4 weeks and litters from poultry farms with a history of poultry contamination with CAP neither contained the drug nor *S. venezuelae*.

Paper 7: A study was conducted to develop and validate a microbiological screening method for multiple antimicrobials namely chlortetracycline, doxycycline, penicillin, erythromycin, tylosin, trimethoprim, ciprofloxacin, enrofloxacin and norfloxacin in eggs, honey, meat and milk samples. Comparisons were made with a standard UK 6–plate method.

Paper 8: A direct competition ELISA method for monitoring tetracycline residues in edible animal products was developed and validated with a limit of detection (LOD) of 10 ng/mL plasma. High levels of antibodies were obtained in rabbits and chicken eggs. Assay results were reproducible although high background reactions were noted.

Paper 9: In another study, six antibodies against CAP were produced in camels, donkeys and goats and evaluated for sensitivity using ELISA and biosensor assays. The effect of matrices and other drugs was also studied. The biosensor method showed limited cross–reactivity compared to ELISA although with reduced sensitivity. Milk samples showed no effect on sensitivity compared to kidney samples.

Paper 10: Another competitive ELISA technique with an LOD of 0.1 ng/mL was developed and optimized for analysis of CAP in sheep serum. The assay was highly specific for CAP and could therefore be used to screen residues of the drug in animal products with limited cross–reactivity due to related analytes.

Paper 11: The pharmacokinetics of CAP was also studied in sheep following intramuscular injection of the drug. A CAP–ELISA technique was then used and the mean maximum

concentration (Cmax), the time taken to reach maximum concentration (tmax), the mean residence time (MRT) and mean elimination half–life calculated. The total body clearance (ClB) obtained was 0.00024 ± 0.000036 mL/h per kg, demonstrating prompt absorption and distribution of the drug following injection.

Paper 12: A radioimmunoassay (RIA) technique was developed, validated and used to screen residues of florfenicol and florfenicol amine in fish muscle in Brazil. Antibodies produced locally (Brazilian antibodies) and others obtained elsewhere (UK) were used. Confirmation of the residues was done using an LC–MS/MS method. The drug residues were detected within the recommended residue levels.

Paper 13: A robust screening method for selected β_2–agonists in bovine liver was studied following European Union (EU) Commission Decision 2002/657/EC criteria. The method detection capability (CCβ), ranged from 0.25 µg/kg to 0.5 µg/kg. The technique was found to be suitable for routine monitoring of the drug residues in beef in Brazil.

Paper 14: A ^{125}I solid–phase radioimmunoassay (RIA) was developed for CAP analysis. Commercially available ^{125}I–labelled streptavidin was selected as the tracer. Biotinylated CAP succinate was chromatographically purified and checked for reactivity to streptavidin in a modified enzyme linked version of the original RIA with streptavidin–horse radish peroxidise as the label. This procedure was found to be feasible and promising, and thus a new concept of ^{125}I solid–phase RIA was developed. This should be applicable for analysis of a range of analytes including CAP. The biotinylation method should be applicable to other analytes as well, such as stilbenes or steroid glucuronides.

Paper 15: LC–MS/MS and HPLC–FLD methods were developed and used to test ten benzimidazoles and avermectin residues in milk for human consumption. Sample preparation involved the QuEChERS method and recovery values were typically in the range 80%–110% with coefficients of variation of < 14%.

Paper 16: In a separate study, an efficient and sensitive LC–MS/MS method was developed for determination of benzimidazole residues in animal tissues and milk. This involved use of $^{13}C_6$–thiabendazole as internal standard. This technique also involved an optimized QuEChERS method and recoveries were between 70% and 110% with coefficient of variation of less than 10%. A corresponding competitive ELISA was established to screen for residues of albendazole and its metabolites in animal foodstuff.

Page 17: A screening method was developed for the analysis of five sulfonamides in chicken meat. Samples were homogenized and extracted with ethyl acetate, the solvent evaporated to dryness, and the resultant residue dissolved in methanol and water before analysis by thin layer chromatography.

Paper 18: Another screening technique (HPLC–DAD) was developed to detect seven sulphonamides in shrimp and chicken tissues. Following extraction and clean–up, chromatographic peaks were detected at 270 nm. Recoveries of the seven sulphonamide residues were in the range 70%–100% at a fortification level of 100 µg/kg.

Paper 19: An HPLC–DAD technique was also developed for the nitrofuran metabolites 3–amino–2–oxazolidinone (AOZ), 5–morpholinomethylmethyl–3–amino–2–oxazolidinone (AMOZ), semi–carbazide (SEM) and 1–aminohydantoin (AHD). Nitrophenyl derivatives (NPAOZ, NPAMOZ, NPSEM and NPAHD) were detected at 275 nm and below 1 µg/kg. Recoveries were above 100%.

Paper 20: How fit an HPLC–UV method is for purpose of analyzing nitrofuran metabolite residues in beef was studied in Namibia. Some good recoveries were obtained and method detection limits were between 2.5 µg/kg and 5 µg/kg. Nitrophenyl products were also synthesized to facilitate easier detection of the analytes by the HPLC–UV and ensure that the method meets the minimum required performance limit of 1 ng/g.

Paper 21: A multi–residue LC–MS/MS method was developed to support the monitoring of AOZ, AMOZ, AHD and SEM in animal products. To improve the method's performance, a number of deuterated drug analogues namely AOZ–d4, AMOZ–d5, AHD–^{13}C3 and SEM–$^{15}N_2{}^{13}$C were used as internal standards. Method validation was done following EU guidelines.

The following conclusions were drawn while closing the CRP D52036:

- A research network was established under this CRP comprising of 17 research organizations from 15 IAEA Member States for 5 years. It provided an opportunity to share methods, process samples and compare results and to strengthen the analytical capabilities of the participant laboratories to develop and validate methods for monitoring contaminants.

- New multi–residue screening/confirmatory methods including use of state–of–the-art LC–MS/MS techniques with stable isotopes (e.g. ^{13}C labelled sulpha compounds) for drug residue analysis in animal products and environmental samples were developed.

- Flumequine pharmacokinetics studies were conducted in sea bream using ^{14}C flumequine involving whole body autoradiography and liquid scintillation counting. The study demonstrated that flumequine has a kinetic profile suitable for use in sea bream and assures consumer safety as the distribution and elimination of the drug were rapid.

- Natural occurrence of the antibiotic Chloramphenicol (CAP) was investigated in animal products and environmental samples (pasture and soil) using a tailored rapid screening technique supported by an LC-MS/MS method. The method was been applied in Mongolia.

- A RIA technique was developed, validated and used to screen residues of florfenicol and its amine analogue in fish muscle in Brazil. A RIA kit was also developed. Method confirmation for this method was done using an LC–MS/MS.

- LC–MS/MS and HPLC–FLD methods were developed, validated and applied to test ten benzimidazoles and avermectin anthelmintic residues in Peru. Innovative extraction of milk samples was employed. A related efficient and sensitive LC–MS/MS method was developed for determination of benzimidazole residues in animal tissues including milk, using ^{13}C–thiabendazole as an internal standard. This method was applied in China.

- Furthermore, a multi–screening thin layer chromatographic method for testing sulphonamide drug residues in chicken tissue was developed and applied in Sri Lanka.

- A robust confirmatory and quantitative LC–MS/MS method was developed for simultaneous determination of sixteen aminoglycoside antibiotics in porcine tissues. This method was also applied in China.

- A multi–parametric Dot ELISA was developed for the detection of enrofloxacin and ciprofloxacin in chicken meat. Specific factors contributing to successful production of antibodies were studied. A complementary accurate, reproducible and highly sensitive LC–MS/MS method for testing enrofloxacin and ciprofloxacin in chicken matrices was also validated. Broiler chickens were successfully used to determine the depletion of enrofloxacin in muscle, kidney, liver and plasma.

- As an offshoot of work that involved use of streptavidin–HRP and ^{125}I–labelled streptavidin as tracer, a direct competition ELISA test was developed and validated for monitoring tetracycline residues in edible animal products with a limit of detection of 10 ng/mL plasma.

- A number of publications were reported in peer review journals.

- Some of the technology developed in the CRP has already been disseminated to laboratories in other Member States through expert missions, fellowships and scientific visits under IAEA's Technical Cooperation program and as SOPs/protocols.

- The CRP also identified new pertinent areas of research such as untargeted analyses that could be filled through future projects. There were a number of spill–overs from the CRP such as attaining ISO accreditation by some participating laboratories and completion of MSc/MPhil degrees through participation under a couple of projects.

- Producing of good antibodies suitable for immunoassays presented challenges.

- As a result of discussions and the preliminary work done on veterinary drugs in fish under the project, and given the growing global aquaculture industry whose safety requires control, a new IAEA CRP was initiated in 2015 on radio–analytical and complementary techniques to control residues of veterinary drugs and related chemicals in aquaculture products.

PAPERS PRESENTED

THE DEVELOPMENT OF MICROBIOLOGICAL MULTI–RESIDUE AND ALLIED LC–MS/MS METHODS TO MONITOR THE VETERINARY DRUG RESIDUES IN THE ENVIRONMENT

KWON, J
Center for Food and Drug Analysis
Busan Regional Food & Drug Administration
Ministry of Food and Drug Safety
[Formerly with Animal, Plant and Fisheries Quarantine and Inspection Agency (QIA)]

Abstract

Microbiological multi–residue screening/confirmatory methods including use of state–of–the-art LC–MS/MS techniques for animal products and environmental samples have been developed, validated and applied. Various combinations and modifications of sample preparation procedures were evaluated to suit local conditions. Target veterinary pharmaceuticals were selected including tetracyclines and their epimers, sulphonamides, penicillins, cephalosporins, macrolides, phenicols, quinolones and aminoglycosides. Stable isotopes such as $^{13}C_6$–sulfamethazine (SMT), $^{13}C_6$–sulfamethoxazole (SMTZ), and $^{13}C_6$–amoxicillin (AMOX) among others were used as internal standards (IS) and the cross-talk phenomenon was also studied. Analyte recoveries above 70% were obtained in environmental samples and leafy plant matrices.

1. INTRODUCTION

Effects of pesticides, organic pollutants and toxic metals on the environment including soil, water, atmosphere, plant, and ecosystem have been widely investigated [1–9]. Pesticides have for long shown great benefit to food security and prevention of infectious diseases. However, their overuse/abuse have also imprinted negatively on public perception [10]. Veterinary drugs are widely used in large quantities to treat animal diseases or promote animal growth and residues of a good number of them are discharged into the environment [11, 12]. Unlike pesticides and other pollutants, the environmental fate, behaviour and potential effects of veterinary drugs are less understood. Foods such as corn, potato, lettuce and carrot uptake antibiotics when grown on soil fertilized with livestock waste [13].

Most researches since the mid to late 1990s have concentrated on the occurrence and distribution of drugs for human and animal in the environment [14]. Various analytical methods based on modern but expensive instruments have been developed to monitor human and veterinary drugs in the environment since the 1990's. Some analytical methods are directly applicable but others are less reproducible for various reasons and would require tailoring to local conditions. Korean scientists have faced similar challenges to their counterparts' in advanced countries during the 1990s regarding residue monitoring namely emerging contaminants, low levels and the need to identify suitable instrumentation and techniques.

Veterinary drugs are newly emerging environmental contaminants in The Republic of Korea and need generation of risk assessment data to support drug registration and to ensure a sustainable environment. The drugs are widely used in large quantities in the country to treat diseases or to promote terrestrial and aquatic animal growth. The Korean terrestrial and aquatic animal farming industries have a very intensive rearing system, with fish farming ranking 14th in the world [15]. Farmers still self-prescribe and treat their animals with drugs without a veterinarian's direction. The use of veterinary medicine as feed additives is also prevalent. The increasing development of antimicrobial resistance and the occurrences of disease outbreaks such as avian influenza, Foot and Mouth Disease and Brucellosis that warrant use of veterinary pharmaceuticals, cause great concern in Korea.

The purpose of this project was also to develop and determine if the microbiological multi-residue screening/confirmatory methods used for analysis of drug residues in animal products can be applied to environmental samples based on LC–MS/MS. Potential sources of inaccuracy in LC–MS/MS methods when using stable isotopes and multi–residue analysis such as cross–talk [16–19) as well as data interpretation [20] were also studied.

2. MATERIALS AND METHODS (ANIMAL FARMING ENVIRONMENT)

2.1.TARGET ANALYTES

Soil, manure, and ground/stream water were selected as major target samples to be analysed for the animal farm environment.

Target veterinary pharmaceuticals were selected by prioritizing on the basis of: annual sales, toxicity, incidents, and some situations of interest in Korea. The pharmaceuticals were tetracyclines and their epimers, sulphonamides, penicillins, cephalosporins, macrolides, phenicols and aminoglycosides including chlorotetracycline (CTC), oxytetracycline (OTC), penicillin (PEN), AMOX, cephalexin (CEPH), tylosin (TYL), erythromycin (ERY), streptomycin (STREP), SMT, sulfamethoxazole (SMTZ), sulfathiazole (STZ), enrofloxacin (ENR), and florfenicol (FFC). For the first phase of this project, two tetracyclines and their epimers, CTC and its epimers 4–epichlor-tetracycline (ECTC), 4–epi–anhydrochlotetracycline (EACTC), OTC and its epimer 4–epioxychlortetracycline (EOTC), 3; the sulphonamides: SMZ, SMTZ and STZ were tested.

2.2. PREPARATION OF REFERENCE MATERIALS

Solid manure samples were collected from 7–10 year old government accredited organic piggery and chicken farms. Liquid manure was prepared by distilled H_2O extraction of solid manure. Solid manure and distilled H_2O were mixed 1:1 (w/w) ratio and shaken for 5 h. Soil of defined physico-chemical properties (Table 1) was prepared from Pesticide Dissipation Test Fields of ChungBuk National University, where veterinary drug and manure had not been applied for more than 10 y.

TABLE 1. PHYSICO–CHEMICAL PROPERTIES OF REFERENCE SOIL

pH (Soil:H_2O,1:5)	Organic content (%)	Cation Exchange Capacity (cmol(+)/kg soil)	Texture (%)			Classification
			Sand	Silt	Clay	
6	0.6	8.4	38.1	61.5	0.4	Silty loam

2.3. MICROBIOLOGICAL MULTI–RESIDUE SCREENING METHOD

For microbial bioassay screening, *Bacillus megarerium* (ATCC 9885), *B. subtilis* (ATCC 6633), *B. stearothermophilus* (ATCC 10149), *B. cereus* (ATCC 11778) as well as *E. coli* (ATCC 11303) were used. The assays included use of *B. megaterium* (ATCC 9885) at pH 7.3 ± 0.1 with 1 mL of a Trimethoprim (TMP) solution added to the medium, *B. stearothermophilus* (ATCC 10149) at pH 6.55±0.05, *B. subtilis* (ATCC 6633) at pH 7.9 ± 0.1, *B. cereus* (ATCC 11778) at pH 5.85±0.05. Sample portions were placed on 5 paper–discs (10 mm diameter) for 30–60 min; each disc was incubated at 45ºC on *B. megaterium* plate, 37ºC on *B. subtilis* and *E. coli* plate, 30ºC on *B. cereus* plate, and 55ºC on *B. stearothermophilus* plate for 16~18 h. After incubation, residues were detected based on zones of inhibition (≥14 mm suggested positive samples).

2.4. LC–ESI–MS/MS

The Acquity UPLC system coupled to TQD and XEVO triple quadrupole MS (Waters) with an electrospray source was used. A C18 Acquity column UPLC BEH (100 mm×2.1 mm; 1.7 µm particle diameter) was used for separation and data processed by Masslynx 4.1 software. HPLC grade water (H_2O), methanol (MeOH), and acetonitrile (MeCN) were used as well as formic acid and ammonium acetate MS grade, Na_2EDTA analytical grade, Celite 545 residue grade. For SPE, Oasis HLB, MCX and MAX cartridges (Waters, Milford, MA, USA), and C18 (NiChroprep RP–18, Merck, Darmstadt, Germany) were used.

3. RESULTS AND DISCUSSION (ANIMAL FARMING ENVIRONMENT)

3.1. MICROBIOLOGICAL MULTI–RESIDUE SCREENING METHOD

The EC four–plate microbial assay test was adopted to analyse tetracyclines. Except for clean water, most of the samples applied onto *B. megaterium* (ATCC 9885*), B. subtilis* (ATCC 6633), *B. stearothermophilus* (ATCC 10149), *B. cereus* (ATCC 11778), and *E. coli* (ATCC 11303) plates showed false positives (Figure 1). However, following application of the sample extraction and clean–up techniques used prior to LC–MS/MS, the false positive tests were reduced.

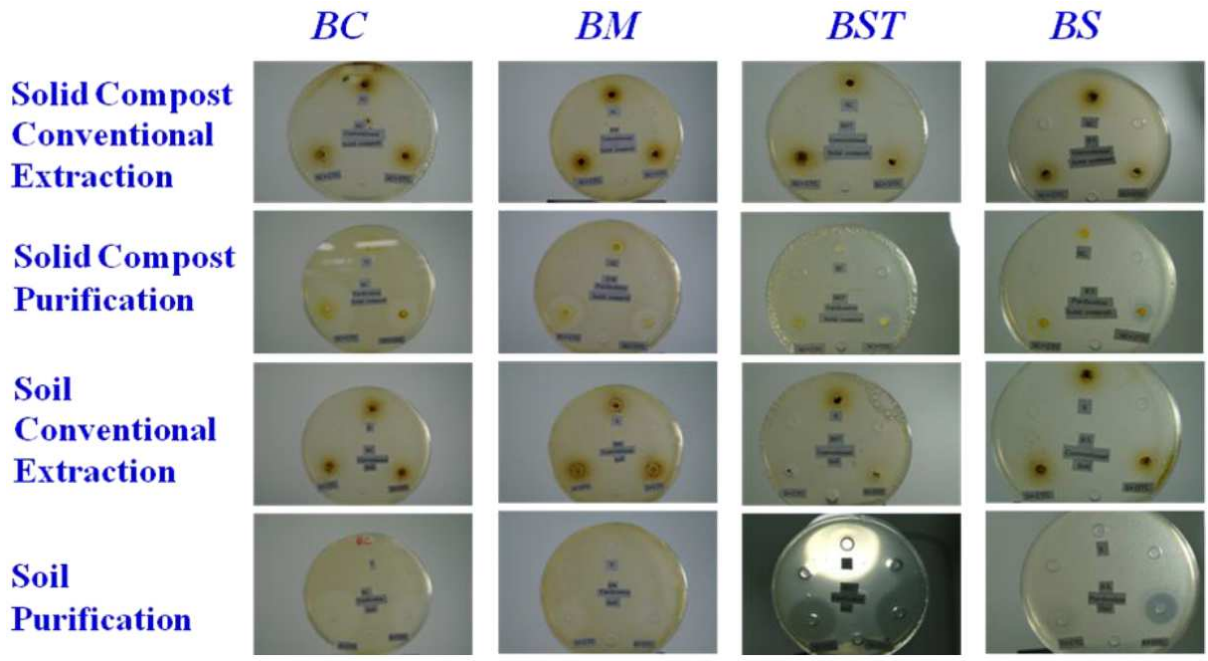

FIG. 1. Microbial bioassays using B. megaterium (ATCC 9885), B. subtilis (ATCC 6633), B. stearothermophilus (ATCC 10149), and B. cereus (ATCC 11778).

Potency tests using different concentrations were performed to evaluate the biological activities of the tetracylines in the study along with their epimers. Fortification was done at levels equivalent to the MRLs in foods. Limited detection capabilities were observed on *B. megaterium* and *B. subtilis* (for TC and CTC) and *B. subtilis* for OTC (Table 2). The biological potency of each epimer was also evaluated against various microorganisms relative to the parent TCs. Based on these tests, more advanced validated microbial screening methods were recommended, and that biologically active epimers may be considered as marker residues for MRL setting in regulatory control purposes.

TABLE 2. THE INHIBITION RESPONSES AGAINST TCs AND THEIR EPIMERS USING EC FOUR–PLATE TEST

Group	Drug	Microorganism	Amount/Diameter (μg/mm)		
			0.1 μg	0.5 μg	1 μg
CTC	CTC	BM	-	-	-
		BC	10 mm	28 mm	32 mm
		BS	-	15 mm	15 mm
		BST	11 mm	30 mm	33 mm
	ECTC	BM	-	-	-
		BC	-	-	-
		BS	-	-	-
		BST	-	-	20 mm
	EACTC	BM	-	-	-
		BC	-	25 mm	-
		BS	-	-	-
		BST	-	29 mm	30 mm
OTC	OTC	BM	-	17 mm	15 mm
		BC	15 mm	22 mm	27 mm
		BS	-	22 mm	18 mm
		BST	-	23 mm	26 mm
	EOTC	BM	-	12 mm	10 mm
		BC	-	20 mm	15 mm
		BS	-	-	-
		BST	-	25 mm	20 mm

* BM; *B. megaterium* BC; *B. cereus* BS; *B. subtilis,* BST; *B. stearothermophilus*

A modified EC4PT method was useful for screening of antimicrobials in the environmental samples such as compost, soil, and stream water. However, further modification is required for other antimicrobials.

3.2. LC–M/MS OPTIMIZATION

Various conditions such as MS compatible solutions, LC column, mobile phase and gradient elution were tested in order to save analysis time and for efficient chromatographic separation. Optimization of the various MS/MS experimental conditions was also carried out by full scan and multiple reaction monitoring (MRM) with direct injection of single and mixed standard solutions. HPLC conditions included C18 Acquity UPLC BEH (100 mm×2.1 mm; 1.7 μm particle diameter; Waters corporation); programmable 20mM NH_4HCO_3/20% MeOH with 20mM NH_4HCO_3/95% MeOH. Each of MRM transitions (m/z) were as follows: CTC 479>444, 479>462; OTC 461>426, 461>443; SMT 279>124, 279>186; SMTZ 254>108, 254>156 (99); STZ 256>108, 255>156 (92). The internal standards (ISs) had the following transitions $^{13}C_6$–SMTZ 260>162, and $^{13}C_6$–SMT 285>124. Meanwhile, CTC, OTC

and their epimers were difficult to separate and identify by MS/MS, while sulphonamide stable isotopes were sometimes shown as interferences especially when chromatographic separation was poor and matrices were complex matrices such as solid manure.

Thus, simultaneous determination of analytes was first carried out, and each major/parent compound, epimer, and stable isotope re–examined. Careful scrutiny was needed when the stable isotope was used as an IS, to determine the epimers/isomers. For simultaneous analysis of AMOX and PEN residues, programmable mobile phase consisting of 0.3% H_2CO_2 + 0.1% NH_4HCO_3/H_2O and MeCN:MeOH was applied. A 5mM of $H_2C_2O_4$/MeCN:MeOH (1:1, v/v), and $H_2C_2O_4/H_2O$ mobile phase was also applied in the Modified US EPA Method 1694. The MS/MS parameters for this study are described in Table 3.

TABLE 3. MS/MS PARAMETERS.

Generic Name	Class	MRM transition (m/z) Precursor ion → Product ion	Cone Voltage (V)	Collision Energy (eV)
CTC	Tetracyclines	479>444, 479>462	36	18, 18
ECTC	Tetracyclines	479>444, 479>462	36	18, 18
EACTC	Tetracyclines	461>154, 461>444	36	18, 18
OTC	Tetracyclines	461>426, 461>443	34	18, 14
EOTC	Tetracyclines	461>426, 461>444	34	18, 14
SSMT	Sulpha drugs	279>124, 279>186	40	26, 16
SMT–6–13C	Sulpha drugs	285>124	36	26
SMTZ	Sulpha drugs	254>108, 254>156 (99)	36	24, 16
SMTZ–6–C–13	Sulpha drugs	260>162	30	16
STZ	Sulpha drugs	256>108, 255>156 (92)	34	20, 14
AMOX	Penicillins	366>114, (366>208), 366>349,	22	20, 8
AMOX–6–C–13		372>214, (372>114), 372>160	22	10, 10
PEN		335>160, 335>176 (335>114, 335>87)	20	10, 15 (35)

3.2.1. Sample extraction and purification

Sample extraction and purification for two TCs, three sulphonamides and two penicillins in prepared veterinary drug–free reference materials was conducted. A simultaneous extraction and purification step was studied taking into consideration matrices and analyte type. For example, TCs chelate and their degradation is pH dependent, while sulphonamides are very stable. Thus, to avoid chelation, and degradation of TCs, EDTA–Mcilvaine buffer (pH 6) was adopted as an extraction solvent while shaking and sonicating.

Filtering was another problem because soil and other matrices have very small particles. To avoid re–adsorption on filtering material, celite 545, less than 1 g of celite was applied after preliminary tests. Also, for all steps plastic ware was used to avoid low recoveries. Clean-up was done using HLB, MCX, and C18. HLB and MCX efficiencies were highly matrix dependent but the more costly C18 showed a general purpose and found to be fit–for–purpose. No degradation was observed when using EDTA–Mcilvaine buffer (pH 4) was observed, perhaps due to short clean-up time.

Recovery ratio of two TCs, and three sulphonamides in water and liquid manure ranged from 79.44% to 116%, and 76.46%~110% for manure compost (Table 4). Reporting limits of TCs and sulphonamides for soil, solid composite, liquid manure/H_2O were <0.05 ng/g, <0.1 ng/g (except SMTZ) and 0.0025 ng/g, respectively. Recoveries of two penicillins varied

(61.71%~90.37%) for solid matrices. However, when the stable isotope, $^{13}C_6$–AMOX was added as an IS, more variable recoveries were obtained for solid matrices.

TABLE 4. MEAN RECOVERY OF VETERINARY PHARMACEUTICALS IN SOIL AND ANIMAL MANURE COMPOST

Generic Name	Soil/compost		Water/liquid samples	
	Mean (%)	CV (%)	Mean (%)	CV (%)
CTC	110	7.5	108	4.3
ECTC	71.27	8.2	78.23	5.4
EACTC	72.32	9.3	76.64	5.7
OTC	92.45	7.3	79.44	7.2
EOTC	80.34	6.4	82.43	6.7
SMT	101	8.4	101	7.3
SMTZ	88.72	8.2	89.53	6.3
STZ	77.51	9.4	81.23	7.8
PEN	90.37	10.2	87.64	11.2
AMOX	61.71	9.8	75.32	8.7

Calibration and recovery test were performed with and without stable isotopes as internal standards.

For the determination of SMT, and SMTZ with $^{13}C_6$–SMT and $^{13}C_6$–SMTZ used as IS, various parameters were adjusted, such as, column type, mobile phase, cone voltage, collision energy and MRM transition, integration parameters. This was also done to correct matrix interferes against quantitation, especially in cases of poorly purified samples. Similar work was done for AMOX with $^{13}C_6$–AMOX. To address interferences and define the applicable scope of isotope use, experiments were done comparing MRM quantification of API LC–MS/MS and exact mass screening of TOF LC–MS/MS.

The decision limit (CCα) and detection capability (CCβ) were estimated according to ISO 11843 using calibration curve, because there's no regulatory guidance or control/permitted limit of target drugs for environment in Korea (Table 5).

TABLE 5. CC ALPHA, CC BETA AND REPORTING LIMIT

Generic Name	CC_α (μg/kg)	CC_β (μg/kg)	Reporting Limit (μg/kg)	Remark (m/z)
CTC	14.9	25.3	<0.05 (soil) <0.1 (Solid Compost)	479>444
OTC	19.2	32.7	<0.05 (soil) <0.1 (Solid Compost)	461>426
SMZ	0.4	0.7	<0.05 (soil) <0.1 (Solid Compost)	279>124
SMTZ	0.5	0.9	<0.05 (soil) N/A (Solid compost)	254>108
STZ	0.5	0.9	<0.05 (soil) <0.1 (Solid Compost)	256>108
AMOX	N/A	N/A	<0.01 (water), <0.33 (Solid Compost)	366>208
PEN	N/A	N/A	<0.01 (water), <0.33 (Solid Compost)	335>160

*CC_α and CC_β were determined from linear regression line of standard calibrations (ISO 11843)

3.3. LC–M/MS OPTIMIZATION

Development of sample preparation and LC–MS/MS technique for residues of TYL, ERY, STREP, ENR, and FFC in the environmental samples were carried out. The developed methods were also applied to monitoring of the same drug residues in the animal farming environment.

Various MS compatible solutions and different elution conditions were tested for possible shorter analysis time and chromatogram separation. Optimization of the various MS/MS experimental conditions was also carried out (full scan and multiple reaction monitoring) following direct injection of individual and mixed standard solutions. Most sample preparation steps were adopted by modification of US EPA Method 1694. The MS/MS parameters for this study are described in Table 6.

TABLE 6. MS/MS PARAMETERS FOR ANALYTES AND INTERNAL STANDARDS

Generic Name	MRM transition (m/z) Precursor ion → Product ion	Cone Voltage (V)	Collision Energy (eV)	Remark
TYL	917>174, 917>772	60	40, 30	
ERY	734>158 734>576 (540)	35	30, 20	ERY-H_2O
ERY–2–C–13	737>578, 737>542	35	20	
STRP	582>263, 582>176	45	35, 38	
ENR	360>245, 360>316	35	25, 20	
CEPHA	348> 158	20	18	
FFC	356>336, 356>185	25	11, 22	

3.3.1. Sample extraction and purification

The samples were extracted in various solvents phosphate buffer, method ethyl acetate and KH_2PO_4 in combination with TCA and purification using C18 SPE. Due to the different physico–chemical properties of the respective drugs, multi–residue analytical methods were not considered proper for environmental samples. Table 7 presents applicability and scope of the developed methods, while figure 2 shows sample chromatogram. Some drugs showed differences in m/z ratios with close retention times. The ratio between precursor and primary ions were thus checked thoroughly to avoid misinterpretation of results. Attention was paid to quality control such as the injection of blank solvents between each injection to prevent carry–over seen for example with FFC. The methods for TYL, ERY, ENR, FFC and STREP were evaluated for recoveries from solid samples (Table 8).

TABLE 7. APPLICATION SCOPE OF DRUG RESIDUE ANALYTICAL METHOD STUDIED UNDER THIS PROJECT

Drug	**Water**	**Solid (soil, manure compost)**	**Purification**	**Separation by HPLC**	**Simultaneous Extraction and Purification**
TYL ERY ENR	Direct	Phosphate buffer extraction	SPE HLB	A; 5mM $H_2C_2O_4$ /MeCN:MeOH (1:1, v/v) B; 5mM $H_2C_2O_4$/H_2O C18 Acquity column UPLC BEH (100 mm×2.1 mm; 1.7 µm particle diameter)	CTC, OTC, PEN, TYL, ERY, SMZ, SMTZ, STZ
ERY TYL	Direct	MeOH extraction	SPE HLB	A; 5mM $H_2C_2O_4$ /MeCN:MeOH (1:1, v/v) B; 5mM $H_2C_2O_4$ /H_2O C18 Acquity column UPLC BEH (100 mm×2.1 mm; 1.7 µm particle diameter)	
FFC	100 mL EtOAc extraction	EtOAc extraction	SPE HLB	A; 70% MeCN B; 80% MeOH C18 Acquity column UPLC BEH (100 mm×2.1 mm; 1.7 µm particle diameter)	

Drug	Water	Solid (soil, manure compost)	Purification	Separation by HPLC	Simultaneous Extraction and Purification
STREP	Direct	KH_2PO_4 +TCA extraction	SPE HLB	A; 10mM HFBA /MeCN:MeOH (3:2, v/v) B; 20mM HFBA/H_2O C18 Acquity column UPLC BEH (100 mm×2.1 mm; 1.7 μm particle diameter)	

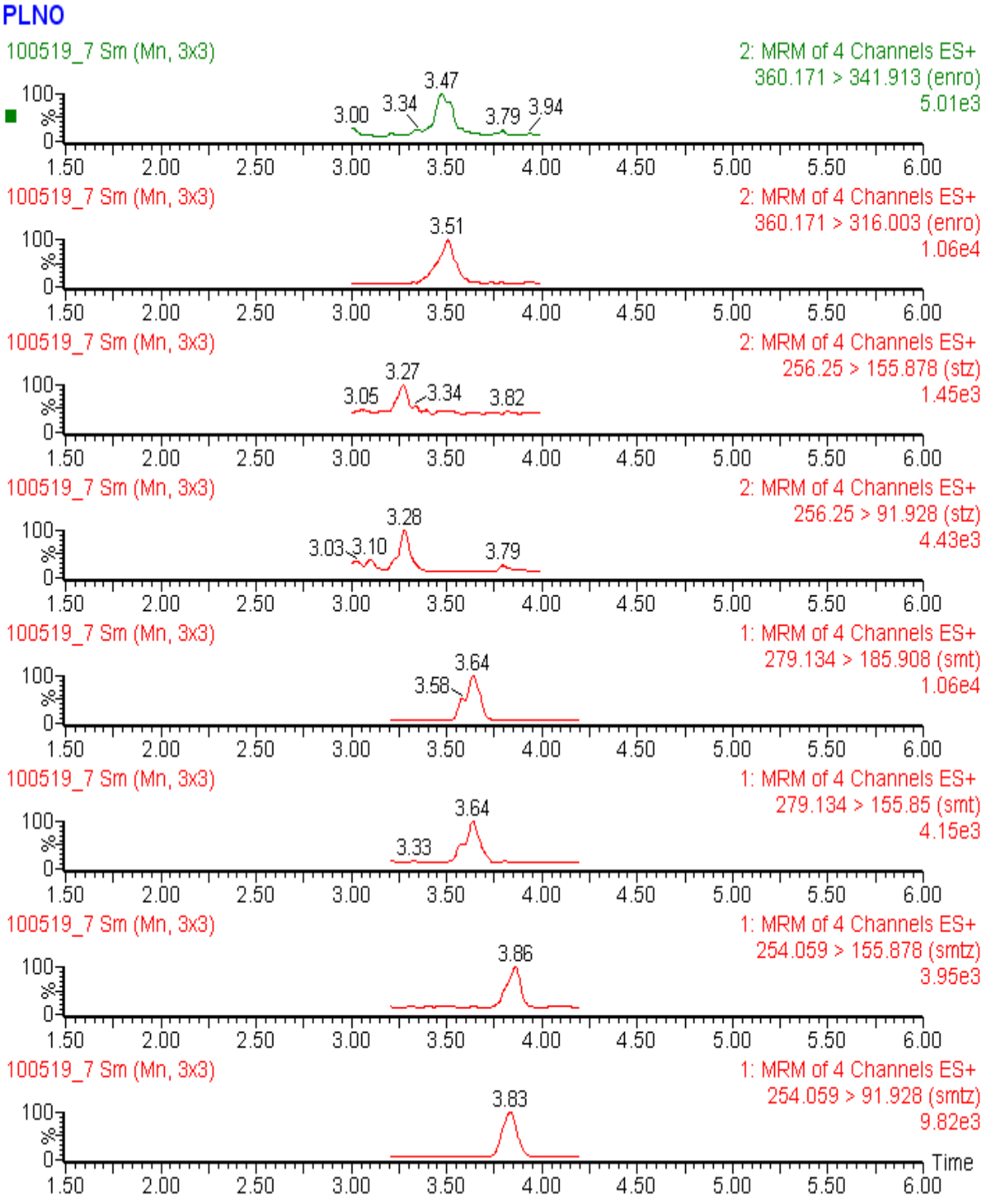

FIG. 2. Sample chromatograms for TYL, ERY, ERY 2–C13, STREP, ENR, CEPHA and FFC (continued).

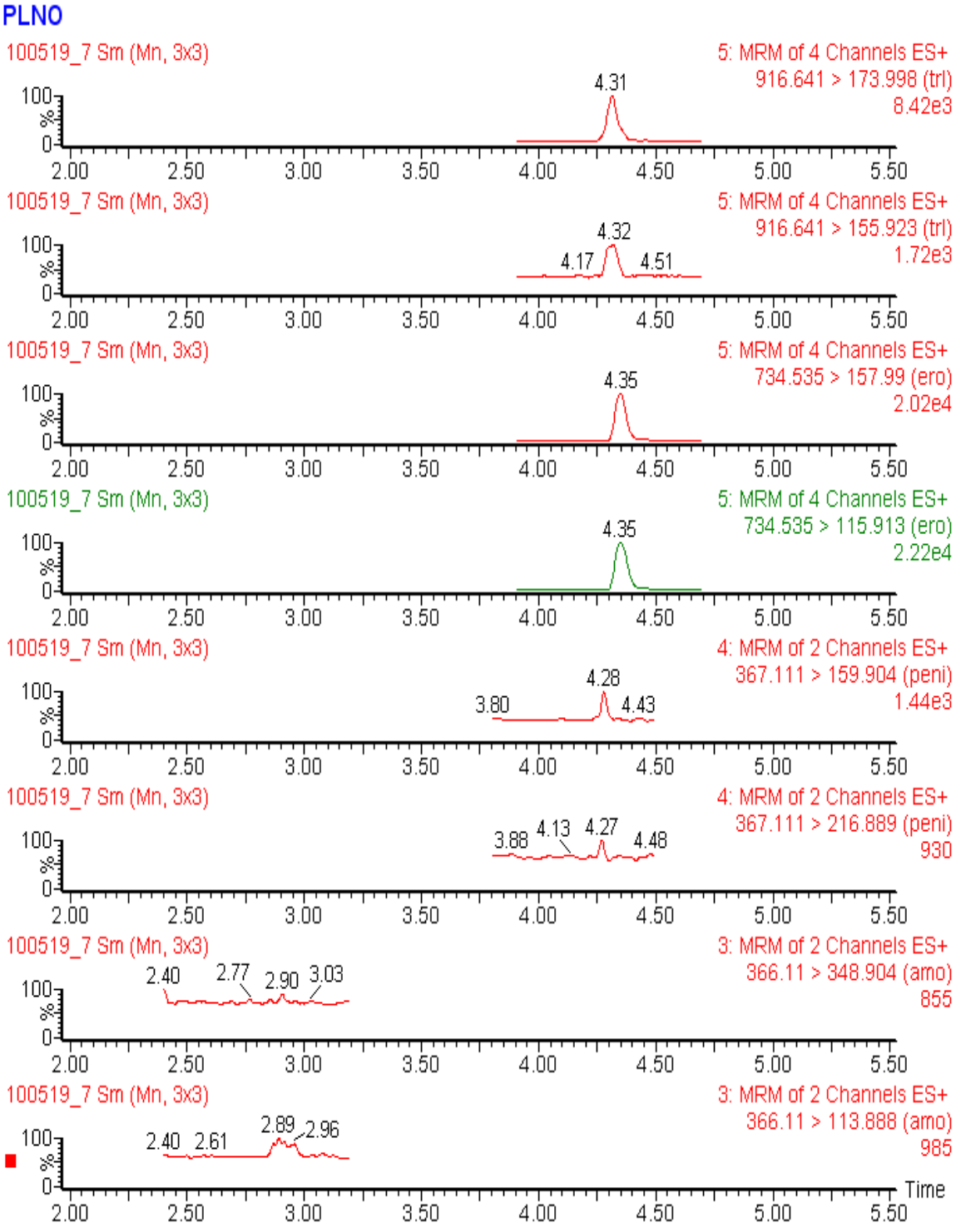

FIG. 2 (continued).

TABLE 8. MEAN RECOVERY VALUES FOR THE VETERINARY PHARMACEUTICALS: TYL, ERY, ERY 2 – ^{13}C, STREP, ENR, CEPHA AND FFC FROM SOIL AND ANIMAL MANURE COMPOST.

Generic Name	Mean (%)
TYL	97.61
ERY	86.00
ENR	79.46
FFC	100
STREP	107

4. APPLICATION OF DEVELOPED METHOD TO A MONITORING PROGRAM OF REAL ENVIRONMENTAL SAMPLES

The developed LC–MS/MS methods were applied in the monitoring program in Korea for two years. Table 9 shows some veterinary drug residue data on environmental samples from animal farms during the first monitoring.

TABLE 9. MONITORING DATA FOR VETERINARY DRUG RESIDUES IN SWINE AND CHICKEN FARMS IN KOREA.

Serial No.	Soil[1]	Solid compost	Liquid compost	Water (Ground)	Remarks (Swine)
Swine–1	0.11 (STZ) 0.17(CTC)	0.3 (STZ) 0.19~0.24 (SMZ) 0.04~0.24 (SMTZ) 0.2~0.5 (OTC) 0.1 (CTC)	0.037~0.042 (STZ) 0.017~0.021 (SMZ) 6.704~6.927 (OTC)	0.008 (SMZ) ~ 0.004 (SMTZ) 0.016 (CTC)	>3000 head Non–HACCP Composting (S,L)
Swine–2		0.24~0.28 (SMZ) 0.01~0.12 (SMTZ) 0.2~0.8 (OTC) 0.1 (CTC)		0.004 (STZ) ~ 0.003 (SMTZ) 0.009 (CTC)	>3.000 head Non–HACCP Composting(S) Ocean dumping
Swine–3	0.06~0.23 (STZ) 0.15~0.18 (SMZ) ~ 0.06 (SMTZ) 0.96~2.05 (CTC)	0.5 (STZ) 0.58~0.60 (SMZ) 0.02~0.17 (SMTZ) 0.3~0.4 (OTC) 2.7~3.3 (CTC)	0.018~0.037 (STZ) 0.121~0.136 (SMZ)	0.004 (STZ) 0.005~0.006 (SMZ) 0.008 (CTC)	>9000 head Previous HACCP Composting (S,L)
Swine–4		0.3 (STZ) 0.56~0.68 (SMZ) 0.1 (CTC)	0.010~0.007 (STZ) 15.886~15.465 (SMZ)	0.004~0.006 (SMZ) 0.008 (CTC)	>5000 head Non–HACCP Composting (S.L)

Soil[1]; Soil around farm, SMTZ; SMTZ, OTC; CTC; PQL (ng/g): H_2O; 0.001, Compost and Soil; 0.03

Farm	Water (ng/g)	Compost (ng/g)	Soil (ng/g)	Remarks
Chicken–1	SMTZ: 323.16 PEN C: 0.01	SMT: <PQL~5.14 SMTZ: 26.93~2558.07 STZ: 2.80~4.38 PEN C: <PQL~0.37 OTC: 88.24 CTC: 207.21 AMOX: 7.19	N/A	>30,000 head Non–HACCP Composting Ground water
Chicken–2	SMTZ: 0.16 PEN C: 0.01 CTC: <PQL	SMT: <PQL~12.07 SMTZ: <PQL~11.05 STZ: <PQL~1.60 PEN C: 0.98~0.51 CTC: <PQL	N/A	>30 000 head Non–HACCP Composting Ground water

Farm	Water (ng/g)	Compost (ng/g)	Soil (ng/g)	Remarks
Chicken–3	STZ: 0.05 OTC: 0.51 CTC: <PQL	SMT: <PQL~4.39 SMTZ: 42.73~433.65 STZ: <PQL~4.52	N/A	>37,000 head Non–HACCP Composting Ground water
Chicken–4	SMTZ: 0.21 STZ: 0.02 PEN C: 0.01 CTC: <PQL	SMT: 4.52 STZ: <PQL~0.90 CTC: <PQL AMOX: 5.00	N/A	>55,000 head/farm HACCP Composting Ground water
Swine–6	SMTZ: 0.38 PEN C: 0.01 CTC: <PQL	SMT: 28.47~2270.23 SMTZ: 2.75~10.60 STZ: <PQL~8.16 PEN C: <PQL~0.61 CTC: <PQL~361.2	N/A	>3,500 head Non–HACCP Composting Ground water
Swine–7	STZ: 0.04 CTC: <PQL	SMTZ: <PQL~17.44 STZ: 39.02~39.657 PEN C: <PQL~0.71 CTC: <PQL	STZ: 177.30	>300 head Non–HACCP Composting Ground water (Food Wastes)
Swine–8	SMT: 0.68 SMTZ: 0.46 STZ: 0.09	SMT: 101.34~1457.96 SMTZ: <PQL~4.16 STZ: 50.49~566.19 PEN C: <PQL~0.36 OTC: 13.19~47.27 CTC: <PQL~51.21	N/A	>130 head Non–HACCP Composting Ground water (Food Wastes)
Swine–9	STZ: 0.03 FFC: 2.81	SMT: <PQL~2.12 SMTZ: <PQL CTC: <PQL	SMTZ: 21.15 STZ: 1.10	>300 head Non–HACCP Composting Ground water (Food Wastes)

Farm	Water (ng/g)	Compost (ng/g)	Soil (ng/g)	Remarks
Chicken–1	ENR: 0.10 FFC: 15.40	TYL: 8.50~14.76 FFC: 41.95	N/A	>30,000 head Non–HACCP Composting Ground water
Chicken–2	ENR: 0.08 FFC: 1.82	TYL: 118.57~583.87 CTC: <PQL FFC: 51.87	N/A	>30 000 head Non–HACCP Composting Ground water
Chicken–3	ENR: 0.13 FFC: 0.50	SMT: <PQL~4.39 SMTZ: 42.73~433.65 STZ: <PQL~4.52 TRL: 7.60~52.85 ERY: 3.83~4.70	N/A	>37 000 head Non–HACCP Composting Ground water
Swine–1	ENR: 0.13 FFC: 1.11	ENR: 7.42~19.99 TYL: 137.66~3488.09	TRL: 13.73	>300 head Non–HACCP Composting Ground water
Swine–2	ENR: 0.17 FFC: 122.62	ENR: <PQL~23.79 TYL: 18.22~1422.88 FFC: 12.89	N/A	>3,000 head Non–HACCP Composting Ground water
Swine–3	FFC: 0.51	ENR: 8.78~15.13 TYL: 457.70~10 418.80 ERY: 5.30~5.39 FFC: 23.14	N/A	>3500 head Non–HACCP Composting Ground water

5. MATERIALS AND METHODS (AQUACULTURE FARMING ENVIRONMENT)

5.1. ANALYTICAL METHOD DEVELOPMENT FOR NINE QUINOLONES IN MARINE WATER BY SPE AND ONLINE–SPE

A method was developed for nine quinolones namely pefloxacin (PER), ciprofloxacin (CIP), norfloxacin (NOR), flumequine (FLU), oxolinic acid (OXL), nalidixic acid (NAL), difloxacin (DIF), ofloxacin (OFL), and enrofloxacin (ENR). The US EPA 1694 method was adopted and modified to improve the sample preparation step. Online–SPE system (nanoACQUITY UPLC 2D system, Waters) with SPE column (OASIS HLB 2.1 × 30 mm, Waters) and SPE (HLB 20 cc/1 g, Waters) were applied for this study.

6. RESULTS AND DISCUSSION (AQUACULTURE FARMING ENVIRONMENT)

Recoveries for each quinolone using on–line SPE were 93.9% (PER), 96.3% (CIP), 89.1% (NOR), 99.9% (FLU), 97.4% (OXL) 95.1% (NAL), 97.5% (DIF), 105% (OFL), and 104% (ENR) (Table 10). However, the values changed to 142%, 71.0%, 92.4%, 73.2%, 115%, 75.4%, 82.6%, 127%, and 105%, respectively when using the conventional SPE. The same LC–MS/MS operational conditions were applied in both cases. This result shows that the online–SPE system is a suitable, reproducible sample preparation step for residue analysis in salt water.

TABLE 10. RECOVERIES OF NINE QUINOLONES WHEN USING ON–LINE SPE AND CONVENTIONAL SPE

Name	On–line SPE	Conventional SPE
PER	93.9	142.0
CIP	96.3	71.0
NOR	89.1	92.4
FLU	99.9	73.2
OXL	97.4	115.0
NAL	95.1	75.4
DIF	97.5	82.6
OFL	105.0	127.0
ENR	104.0	105.0

7. MATERIALS AND METHODS [ANALYTICAL METHOD DEVELOPMENT FOR ERY, LINCOMYCIN (LIN) AND TMP IN MARINE WATER USING SPE AND ONLINE–SPE FOR CLEAN–UP)]

Residue analytical methods of three antimicrobials, ERY, LIN and TMP for fish farming were compared based on conventional SPE and online–SPE clean–up of marine water. The US EPA 1694 method was adopted and modified to enhance sample preparation. On–line SPE system (nano–ACQUITY UPLC 2D system, Waters) with two SPE columns, OASIS HLB (2.1×30 mm, Waters) and HLB (20 cc/1 g, Waters) was used.

8. RESULTS AND DISCUSSION (RESIDUES IN MARINE WATER)

The recovery ratios (%) were 101% (ERY), 93.6% (LIN), and 89.4% (TMP) when using on-line SPE. However, when conventional SPE was employed, these dropped: 0.0% (ERY), 66.6% (LIN), and 68.8% (TMP) (Table 11). Analysis was done by LC–MS/MS. The on–line

SPE system in this regard was more stable and effective sample preparation tool for salt water compared to conventional SPE (Table 12).

TABLE 11. RECOVERIES OF ERY, LIN AND TMP USING ON–LINE SPE AND CONVENTIONAL SPE CLEANUP.

Name	On–line SPE	Conventional SPE
ERY	101	0.0
LIN	93.6	66.6
TMP	89.4	68.8

8.1. ANALYTICAL METHOD APPLICATION

Analytical methods were setup to monitor residues of OTC, OFL, ENR, PER, CIP, NOR, FLU, OXL, sulfadiazine (SDZ), sulfadimethoxine (SDMX), FFC, ERY, CEPHA, and ampicillin (AMP) in the marine aquatic system. The ranges of practical quantitation limit (LOQ) were set from 0.0005 ng/L or ng/g to 20 ng/L or ng/g, and the recoveries ranged from 85.73% to 100% with below the acceptable coefficient of variation of reproducibility (Table 13). Figure 3 presents the results of the antimicrobial residue concentrations in sea water, sea weed, sediment, adhesion organism and feed used in marine fish farming environment. The frequency of occurrence of veterinary drug residues in fresh–water aquaculture system determined using the developed methods is also reported.

TABLE 12. ON–LINE SPE AND LC/MS/MS CONDITION FOR CONCENTRATION AND ANALYSIS

	Separation	Concentration
Mobile Phase	A: 0.01% formic acid in H_2O B: 0.01% formic acid in MeCN flow rate: 0.4 mL/min	A: Loading Solution: water B: Washing Solution: MeOH/aceton/n–hexane = 1:1:1 (v/v/v) flow rate: 2 mL/min
	Analysis	Concentration
Column	UPLC BEH C18 2.1×100 mm, 1.7 µm, Temp 40 °C; Oasis HLB, 2.1× 30 mm, 20µm	
Analysis Time	17 min (injection and concentration: 6 min, analysis: 11 min)	
Injection Volume	5,000 µL, needle wash 1: 10% MeOH, needle wash 2:MeOH	
MS/MS Condition	Capillary voltage: 2 kV Source temp 150°C Desolvation temp 400°C Desolvation gas flow: 800 L/h	

Separation					Concentration			
Time (min)	Flow rate (mL)	%A	%B	Curve	Flow rate (mL)	%A	%B	Curve
	0.4	95	5	11	2	100	0	11
3.8	0.01	95	5	11	0.01	100	0	11
4.1	0.4	95	5	11	2	10	90	11
7	0.4	5	95	6	2	10	90	11
9	0.4	5	95	6	2	10	90	11
10	0.4	95	5	6	2	10	90	11
11	0.4	95	5	11	2	100	0	11

TABLE 13. RECOVERY AND CV OF THE ANALYTICAL METHOD FOR MONITORING

	Sea water (%)	CV (%)	PQL (ng/L)
OTC	100.00	11.83	0.0005
Doxycycline	100.00	7.84	0.0005
OFL	99.98	8.35	0.0005
ENR	100.00	5.73	0.0005
PER	100.00	8.30	0.0005
CIP	100.00	8.56	0.0005
NOR	99.98	11.30	0.0005
FLU	100.00	5.25	0.0005
OXL	100.00	4.32	0.0005
SDZ	100.00	26.75	0.0005
SDMX	100.00	6.00	0.0005
FFC	100.00	6.53	20
ERY	95.65	7.23	0.002
CEPHA	86.94	8.97	0.5
AMP	85.73	6.43	0.1

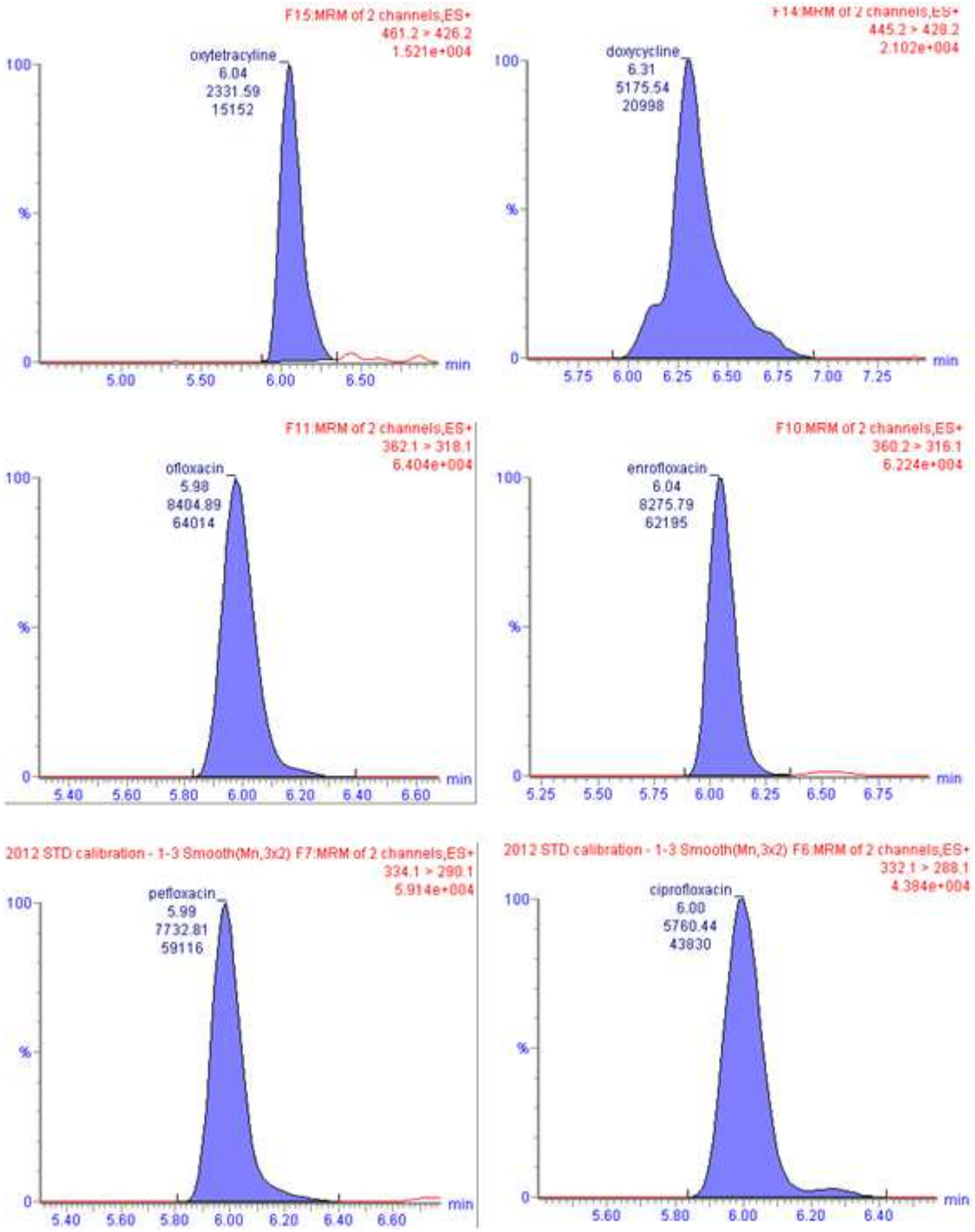

FIG. 3. Chromatograms of target analytes fortified in the seawater at the practical quantitation levels (continued).

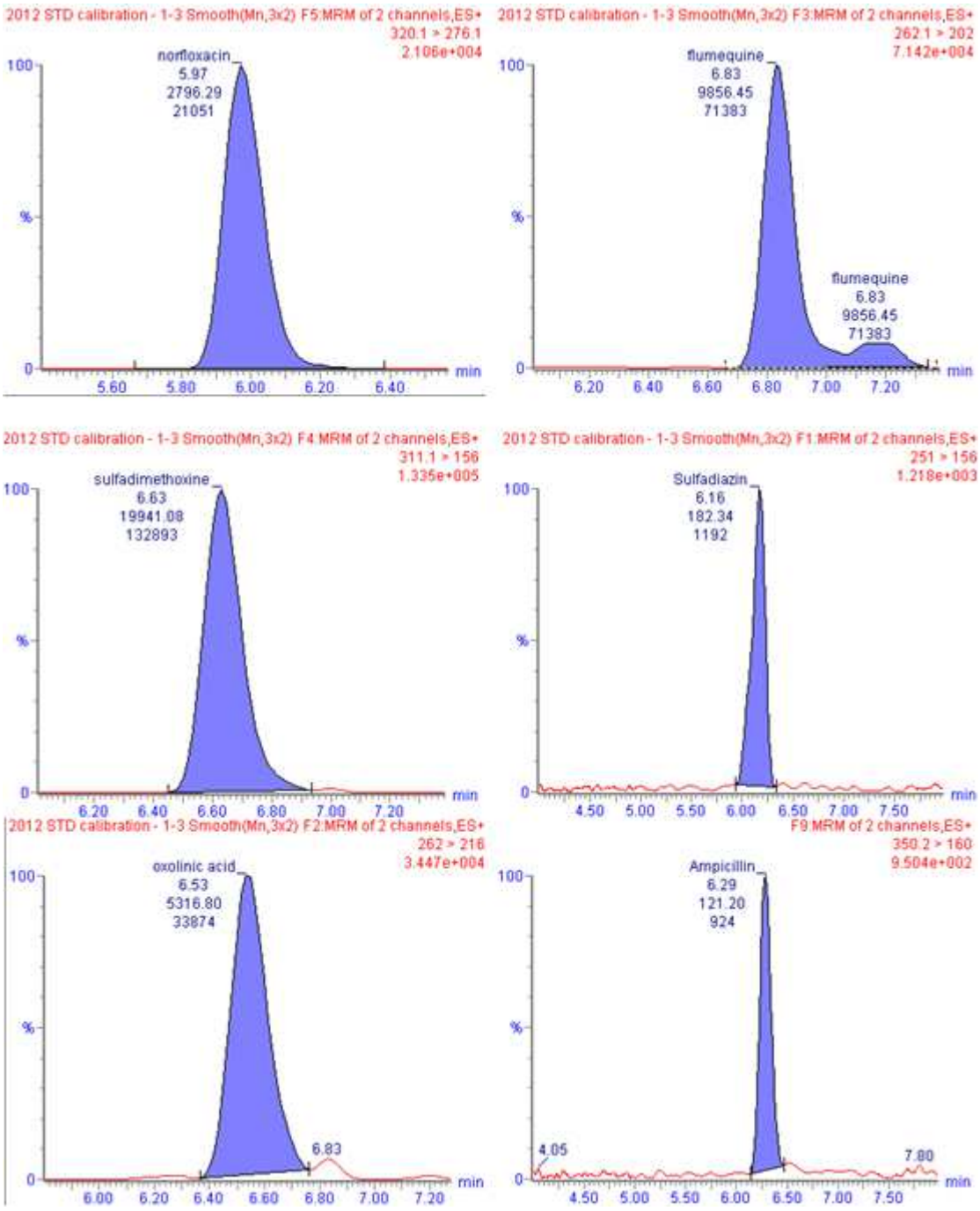

FIG. 3. Continued.

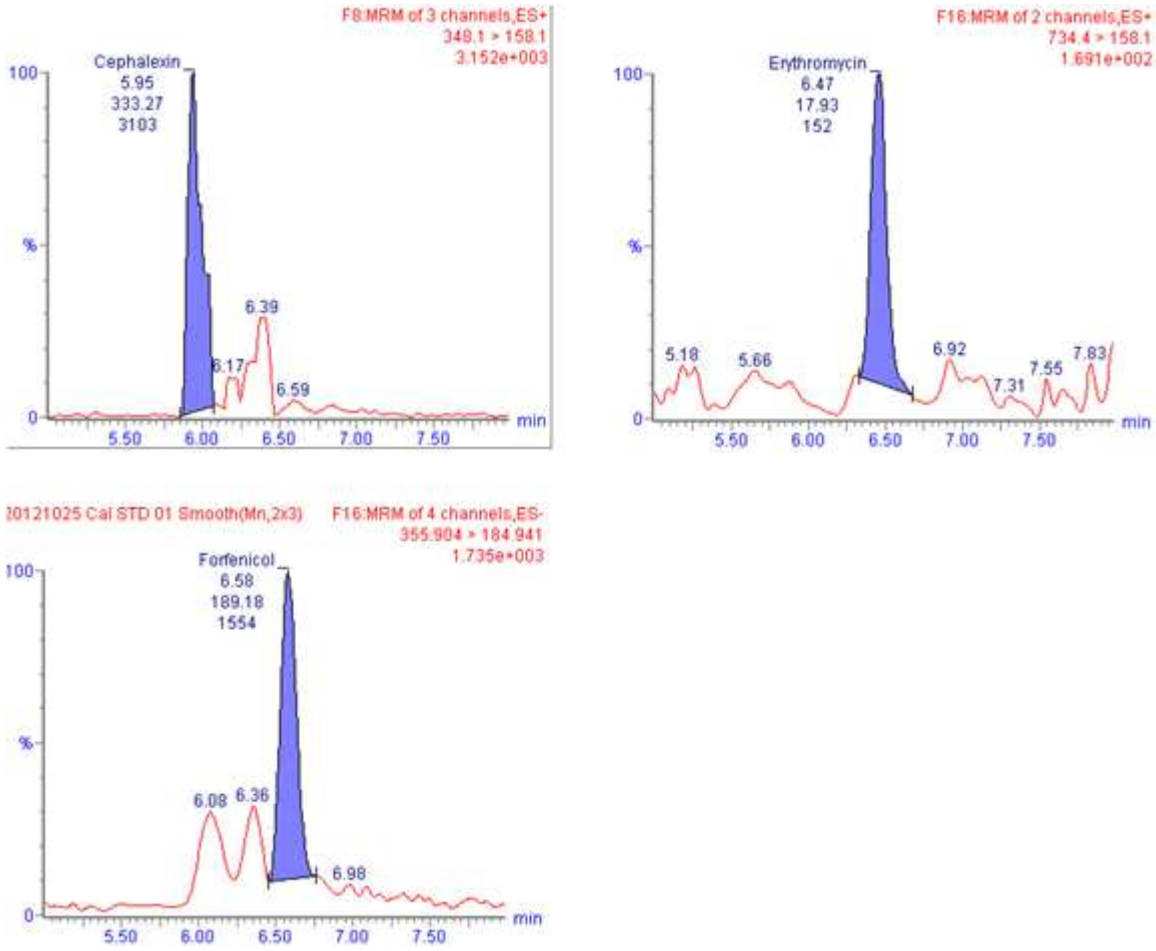

FIG. 3. Continued.

9. MATERIALS AND METHODS (ANALYTICAL METHOD DEVELOPMENT FOR MONITORING OF 14 SULFONAMIDES IN MARINE AND FRESH AQUACULTURE SYSTEM)

The target sulphonamides were SDZ, STZ, sulfamerazine (SMRZ), SMT, sulfamethoxypyridazine (SPDZ), sulfachlorpyridazine (SCPDZ), sulfamonomethoxine (SMMX), SMTZ, sulfadoxine (SDX), sulfisoxazole (SXL), sulfaclozine (SCZ), sulfaphenazole (SPZ), sulfadimethoxine (SDMZ), and sulfaquinoxaline (SQX). Automated on–line sample concentration and purification were performed using UPLC® with On–line SPE coupled to XEVO TQ–MS controlled by Masslynx software version 4.1 (Waters, Milford, MA, USA). Concentration and extraction were performed by Oasis HLB (2.1×30 mm; particle diameter 20 µm) with 2% formic acid/H_2O and 0.5% NH_4OH/MeOH elution. A C18 ACQUITY UPLC BEH (100×2.1 mm; particle diameter 1.7 µm) from Waters and a programmable mobile phase of 0.1% formic acid/H_2O and 0.1% formic acid/MeCN were used. The run time was 16 min including 6 min of concentration after 2 µL sample injection. The LC–MS/MS was used in the positive ionization mode and MRM.

10. RESULTS AND DISCUSSION (ANALYTICAL METHOD DEVELOPMENT AND AQUACULTURE SYSTEM)

Under optimal conditions (Table 14), the PQL set were at ≤0.01 µg/mL for freshwater and seawater. The recoveries of the 14 sulphonamides from seawater were in the range of 94%–108% with the repeatability of 7%. In case of freshwater, the recoveries ranged from 89% to

112% with CVs of 8%. One gram each of swine and fish muscle, fortified at 0.5×MRL and MRL levels of sulphonamides was extracted with 10 mL of MeCN by centrifugation and filtered before on–line SPE. The recoveries/CVs for swine muscle and fish muscle were 85%–126%/2–28% and 82%–144%/2–10% with the same PQL of water samples, respectively (Table 15).

TABLE 14. SUBSTANCE SPECIFIC MS/MS PARAMETERS FOR THE 14 SULFONAMIDES: PRECURSOR, PRODUCT ION (m/z) and COLLISION ENERGY.

Sulphonamide	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)	Product ion (m/z)	Collision Energy (eV)
SDZ	251	92	27	156, 108	15
STZ	256	92	25	156, 108	15
SMRZ)	265	92	28	156, 108	15
SMZ	279	186	16	92, 156	28
SPDZ	281	156	15	92, 108	30
SCPDZ	285	156	15	92, 108	28
SMMZ	281	156	22	92, 108	35
SMTZ	254	156	16	92, 108	26
SDX	311	156	15	92, 108	32
SXL	268	156	12	92, 108	24
SCZ	285	156	14	92, 108	26
SPZ	315	158	28	92, 108	34
SDMZ	311	156	20	92, 108	32
SQX	301	156	16	92, 108	30

TABLE 15. RECOVERIES AND CVS OF SULFONAMIDES IN ENVIRONMENTAL SAMPLES WHEN AN AUTOMATED ON–LINE SPE and LC–MS/MS WERE USED

Sulphonamides	Sea water (MRL)		Sea water (0.5MRL)		Fresh water (MRL)		Fresh water (0.5MRL)	
	% Recovery (±SD)	CV (%)	% Recovery (±SD)	CV (%)	% Recovery (±SD)	CV (%)	% Recovery (±SD)	CV (%)
SDZ	102.00 (2.1)	2.04	95.77 (3.2)	3.38	89.24 (2.9)	3.23	90.85 (1.0)	1.05
STZ	104.00 (2.9)	2.69	97.71 (4.0)	4.05	97.07 (2.0)	2.06	98.54 (3.5)	2.30
SMRZ	104.00 (1.9)	1.83	97.31 (2.2)	2.27	97.79 (1.6)	1.64	96.28 (3.5)	3.60
SMZ	106.00 (1.9)	1.76	98.28 (2.0)	2.07	97.87 (1.7)	1.72	101.00 (1.8)	1.78
SPDZ	107.00 (3.7)	3.45	99.63 (6.8)	6.78	104.00 (3.6)	3.43	113.00 (5.8)	5.12
SCPDZ	105.00 (2.4)	3.74	96.89 (3.6)	2.31	108.00 (3.7)	3.40	110.00 (2.3)	2.08
SMMZ	109.00 (3.0)	2.80	97.43 (3.3)	3.36	98.80 (5.9)	6.03	96.90 (8.7)	8.43
SMTZ	104.00 (1.2)	1.17	98.52 (2.5)	2.64	101.00 (1.7)	1.67	103.00 (1.6)	1.57
SDX	103.00 (1.9)	1.89	98.51 (2.7)	2.76	99.07 (2.0)	2.01	103.00 (3.0)	2.90
SXL	104.00 (2.0)	1.96	95.68 (3.4)	3.59	98.88 (1.3)	1.30	101.00 (1.5)	1.45
SCZ	105.00 (3.5)	3.34	98.48 (7.2)	7.27	103.00 (3.3)	3.25	104.00 (3.3)	3.19
SPZ	104.00 (2.7)	2.39	96.68 (3.3)	3.37	103.00 (1.8)	1.74	104.00 (2.4)	2.30
SDMZ	105.00 (3.0)	2.90	95.46 (2.6)	2.76	105.00 (0.8)	0.76	105.00 (2.5)	2.42
SQX	107.00 (2.9)	2.71	94.85 (3.1)	3.31	104.00 (2.4)	2.30	107.00 (2.4)	2.28

11. INTERPRETATION OF LC–MS/MS DATA AND PREVENTION OF FALSE–POSITIVE RESULTS DUE TO CROSS–TALK

Cross–talk is when the fragment ions from one MRM (SRM) transition are scanned out during another transition and may be associated with false positive results [16–19].

11.1. CROSS–TALK ATTRIBUTED TO STABLE ISOTOPE AS INTERNAL STANDARDS

11.1.1. Qualification

The molecular weight differences between analytes and their stable isotope labelled chemical analogues, is very small, and the chromatographic retention times are almost the same. Even at MS resolution higher than 10, 000, it may not be easy to distinguish between the two chemicals, depending on factors such as operator's experience, analytical condition, instrumental condition among others [21]. Tables 16–18 demonstrate cases of cross talk.

TABLE 16. PRECURSOR AND PRODUCT IONS FOR THE IDENTIFICATION OF CROSS–TALK ASSOCIATED WITH THE IDENTIFICATION OF CLENBUTEROL (CLEN), ERYTHROMYCIN (ERY) AND THEIR STABLE ISOTOPE LABELLED ANALOGUES BY LC–TOF ANALYSIS [21]

Chemical	Precursor ion (m/z)	Product ion (m/z)
CLEN	277 259[M–H_2O+H]+	259,203,168,132,57
CLEN–D9	286	204,268
CLEN–D6	265[M–H_2O+H]+ 283, 285	204,265
ERY	734	576
ERY anhyd.	716	158
ERY–2C–13	736	160
ERY anhyd–2C–13.	718	160

TABLE 17. MS/MS PARAMETERS FOR AMOXICILLIN AND ITS INTERNAL STANDARD [21].

Generic Name	MRM transition (m/z) Precursor ion → Product ion	Cone Voltage (V)	Collision Energy (eV)
AMOX	366>114, (366>208),366>349,	22	20, 8
AMOX–6–13C	372>214, (372>114),372>160	22	10, 10

TABLE 18. PRECURSOR AND PRODUCT IONS OF 14 SULFONAMIDES [21].

Chemicals	Precursor ion (m/z)	Product ion (m/z)
SCPDZ	285	92, 108, 156
SDZ	251	92, 108, 156
SDMZ	311	92, 108, 156
SPDZ	281	92, 108, 156
SMZ	265	110, 156, 172
SMZ	279	92, 156, 186
SMTZ	254	92, 108, 156
SMMZ	281	92, 108, 156
STZ	256	92, 108, 156
SQX	301	92, 108, 156
SXL	268	92, 108, 156
SDX	311	92, 108, 156
SPZ	315	92, 108, 158
SCZ	285	92, 108, 156

12. CONCLUSION

Multi–analyte screening and confirmatory analytical methods including use of stable isotopes were developed for analysis of residues of veterinary drugs in animal products and the environment. The drugs included tetracyclines and their epimers, sulphonamides, penicillins, cephalosporins, macrolides, amphenicols, quinolones and aminoglycosides. The stable isotopes such as $^{13}C_6$–sulfamethazine, $^{13}C_6$–sulfamethoxazole, and $^{13}C_6$–amoxicillin among others were used as internal standards. The phenomenon of cross–talk and its effect on specificity was also studied as well as method accuracy where recoveries above 70% were determined.

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PHARMACOKINETICS OF FLUMEQUINE IN FARMED SEA BREAM (*Sparus auratus*) USING LABELED DRUG

MANSOUR, A. B*., GUERBEJ, H**., BAKKE, M***., SADOK[+]., BERRICHE, Z*., SAMAALI, M*., SHAIKH, B[++]., SASANYA, J[+++]., HORSBERG, T**

* Laboratory of Radiochemistry, National Center for Nuclear Sciences and Technology, Tunisia.
** Aquaculture Laboratory, National Institute for Marine Sciences and Technologies, Monastir.
*** Department of Food Safety and Infection Biology, Section of Pharmacology and Toxicology, Norwegian School of veterinary Science, Oslo, Norway.
[+] Laboratory of quality and enhancement of aquatic products, National Institute for Marine Sciences and Technologies, La Goulette, Tunisia.
[++] Division of Residue Chemistry, Office of Research/CVM, USFDA, USA
[+++] International Atomic Energy Agency, Vienna, Austria.

Abstract

Flumequine (FLU) is one of the main antibacterial agents widely prescribed to treat bacterial pathogens in Tunisian fish farming. A study was conducted to evaluate the pharmacokinetic properties of FLU following a single intraperitoneal administration of 12 mg/kg, corresponding to 100 µCi/kg in sea bream (*Sparus auratus)* interperitoneally as a single dose. The kinetics studies were performed on fish with an average weight of 147 g and held in sea water (salinity 38‰–39‰) at 28°C ± 1°C. Three fish were collected at various times ranging from 0.5 h to 168 h post administration. The absorption, distribution and elimination of ^{14}C–labelled FLU were studied using whole body autoradiography (WBA) and liquid scintillation counting (LSC) and the concentration of FLU equivalent versus time had been evaluated in the major organs. An agreement between the data obtained from the two combined techniques was observed. Rapid and extensive distribution of FLU to the major organs half hour after dosing was recorded. The level of the drug declined in some tissues one hour after dosing, perhaps due to renal excretion after exposure. The fluctuation had been recorded through time in the excretory organs due to the elimination of the drug from other tissues through blood. From 72 h post administration, traces of the drugs were observed in the major tissues of the fish. The results indicate that FLU has a kinetic profile suitable for the selected specie because both the distribution and elimination of the drug were found to be rapid.

1. INTRODUCTION

The aquaculture industry has grown dramatically, and plays an important role in the world's food supply chain. Disease problems constitute the largest single cause of economic losses in this field. Control and treatment options using vaccines and drugs are increasing as a remedy for the production rate. Recently, there has been global attention in antimicrobial resistance in aquaculture, and in controlling antimicrobial resistance through risk analysis and the establishment of regulations and guidelines. Despite the controls and regulations introduced by governments to prevent environmental risks in many countries, antimicrobial resistance and toxicity are increasing. Several international organizations have produced recommendations on the responsible and prudent use of antimicrobial agents in veterinary medicine to reduce the overuse and misuse of antimicrobials in aquaculture in order to protect public health [1].

The drug FLU is a second generation quinolone antibacterial. It is moderately lipophilic and has the properties of a weak acid [2]. It's one of the most common drugs prescribed for the effective control of infectious diseases in fish husbandry caused by a wide-range of gram negative bacteria (*Escherichia coli*, *Salmonella* and *Pasteurella*) [3, 4].

In Tunisia, FLU is one of the main solutions for infectious diseases in fish farming. Its Maximum Residue Limit (MRL) in Tunisia meets the requirement of the European Union Council Regulation on Veterinary Drug Residues fixed at 600 µg/kg in muscle and skin of fish at natural proportion. To apply the correct agent and the optimal dosage regime for

successful treatment, minimize environmental hazards and ensure food safety, knowledge of the pharmacodynamic and pharmacokinetic properties of the FLU in farmed fish is an important tool [5].

Several studies on the pharmacokinetic of FLU in fish have been published including various species, different experimental protocols and environmental conditions. Thus, different pharmacokinetic profiles of FLU in fish species exist. However, since there are no consistencies in experimental design, environmental conditions or modelling techniques, it is difficult to compare the results. Several studies showed that the kinetic profile of FLU demonstrates interspecies differences [6] an indication that the extrapolation of treatment regimes and withdrawal times for target fishes using data derived from other species is improper and may result in false therapies and environmental pollution. These data suggest that the estimation of a withdrawal period following FLU administration in fish requires individual study for each species under defined environmental and dosage conditions [7]. The aim of the present study was to provide additional information on the pharmacokinetic properties of FLU in the gilthead sea bream (*Sparus auratus)* one of the main exported farmed species in Tunisia.

2. MATERIALS AND METHODS

2.1. PURITY TEST OF LABELED FLU

The labeled drug used in the conducted study was ^{14}C–FLU provided from the US Food and Drug Administration and custom synthesized by NEN Life Sciences Products (now Perkin–Elmer) in 1999. Assays had been conducted to test its purity and the HPLC and LSC were the main tools used for this work. First, an HPLC/UV method was adopted including use of a cold FLU. Standard solutions were dissolved in MeOH and then in mobile phase. For three replicates of the working solutions, the eluted ^{14}C–FLU was collected before adding ionic fluor and activity counted using a 1450 Microbeta Trilux LSC. The specifications of the FLU standards, reagents and apparatus as well as the analytical parameters adopted and the data recorded are detailed in Annex I. The radiochemical purity was 94.7% and thus the ^{14}C–FLU was used directly for the kinetics studies.

All consumables used were transferred to the radioprotection section in the CNSTN responsible for radioactive waste management.

2.2.ADMINISTRATION OF LABELED FLU

2.2.1. Test facilities and test fish

The administration step was performed at the aquaculture laboratory, the National Institute for Marine Sciences and Technologies in Monastir, Norway. The sea bream fish had been caught from Monastir coasts and held in 1,000 L reinforced polyester tanks supplied with running sea water at ambient temperature, pH 8.01, salinity 38‰–39‰, Oxygen of more than 3 mg/L). During the 15 days adaptation period the fishes (~15 m old with mean weight of 147.8 g ± 28.8 g) were fed a commercial pelleted fish diet using automatic feeders.

2.2.2. Intraperitoneal (IP) administration

The ^{14}C–FLU was administered IP at a dose of 100 μCi/kg, corresponding to 12 mg/kg after addition of cold FLU. The FLU was first dissolved in NaOH 1M then diluted in saline and the pH adjusted to 10. The injection volume was 2 mL/kg [8, 9]

During the adaptation period the fish were fed a commercial pelleted fish diet. Feed was withheld two days prior to drug administration and during the studies. In each group given the FLU IP, each fish was netted from the acclimatization tank, anaesthetized with phenoxyethanol (30 µL/L, H_2O) and individually weighed. The IP injection was accomplished by placing the fish on a damp towel and the FLU solution slowly/carefully injected into the abdominal cavity using a 1 mL disposable syringe. Each fish was dosed according to individual weight. Before administration, the test suspension was mixed for 15 s on a vortex mixer and a dose aspirated. After drug administration, all dosed fish were placed in individual tanks for observation of any problem before being transferred to experimental tanks. During the studies the temperature was 28°C±1°C and tanks were placed under controlled light conditions corresponding to photoperiod of 14 h of light and 10 h darkness.

2.2.3. Sample collection

In the IP administered groups, samples were collected at 0.5 h, 1 h, 2 h, 4 h, 7 h, 24 h, 48 h, 72 h, 96 h, 144 h and 168 h post administration as recommended elsewhere [10, 11] During each period 4 fish samples were sacrificed by an overdose of phenoxylethanol (0.3 mL/L) and placed in individual plastic bags before being stored at -80°C.

For WAB, each piece was embedded in individual blocks of cooled 1% solution of methylcellulose in water followed by immediate freezing with dry ice (-75°C). Sagittal sections (20 µm) from the whole fish were obtained on tape (No.821, 3M Co., St. Paul, MN, USA) at -20°C in PMV cryomicrotome (PMV.450 MP, Stockholm, Sweden). The sections were freeze-dried overnight, before application on Structurix D7 films for autoradiography. The films were exposed at -20°C for 2 m before developing [8, 9].

From the material remaining in the frozen blocks, samples weighing 10 mg to 100 mg obtained from muscle (red and white), brain, blood, kidney, liver, bile, heart, intestine and spleen were digested with soluene at 37°C. Then 10 mL of hionic fluor were added to each vial. The radioactivity in the samples was counted in a Packard Tri–Carb 1900 CA counter. Three pieces from each sampling points were dissected and organs from each piece treated similarly. The radioactivity in the samples was counted and the concentration of FLU equivalents (radioactivity) versus time in different organs recorded to evaluate the distribution and elimination rate of the drug on fish tissues [8, 9].

3. RESULTS AND DISCUSSION

From the autoradiograms developed a central distribution to most organs through blood was observed at half hour after dosing with large portion of radioactivity in the intestines and gut. One hour after dosing increased radioactivity was recorded in the kidney. At 4 h after dosing, the radioactivity was lower (except intestines) compared to the earlier sampling points. At 7 h after dosing the radioactivity was much lower in all organs. From 72 h after dosing reduced amounts of radioactivity were observed in bile, intestines and skin and nothing observed in other tissues [12].

From the counts recorded with the Perkin Elmer Tricarb, the concentration of FLU equivalent was calculated and graphs involving the variation of the drug concentration through time were drawn. A rapid absorption of the drug by the muscle and brain followed by rapid elimination was observed, with only traces of the drug seen 72 h after dosing. A decrease in some tissues at 0.5 h and 01 h after dosing was attributed to renal excretion post exposure. A fluctuation was recorded in the heart, kidney, and liver perhaps due to the elimination of the drug from

others tissues through blood. Also, fluctuation had been recorded in the intestine raising the possibility of the enterohepatic circulation. The data obtained was modelled for the determination of the pharmacokinetics parameters for comparison with others species [12].

An agreement between the data obtained from the two combined techniques (LSC and WAB) was observed. There was rapid elimination of FLU under seawater holding conditions possibly due to chelation (with $Mg^{2+}$and Ca^{2+}) since sea bream is an active seawater drinker. It could also be due to rapid metabolism of FLU by glucuronidation, a process shown to be more rapid in seawater. A single intake was chosen to evaluate the depletion of the drug in the fish body in this study [12].

Flumequine was licensed for application in farmed fish against systemic bacterial infections such as furunculosis, vibriosis, and enteric red–mouth disease at a rate of 12 mg/kg body weight per day in a feed mixture for 5 days [13]. Previous studies have pointed out that the kinetic behaviour of antimicrobial agents varies considerably between different species [14–16].

A comparative depletion between seawater fishes and fresh water showed that FLU elimination is more rapid in seawater [17]. Some studies were performed in Atlantic salmon reared in seawater where the absorption of FLU from the gastrointestinal tract reached a level 40%–45% of the administered dose at 5°C [18]. Distribution was higher in skin and liver compared to muscle [19] and the drug was retained in bone to a greater extent than in skin [20]. For the same species, more information about the depletion of FLU was published by [18, 21–23]. Ample information on the pharmacokinetics of FLU in other species had also been published.

Flumequine has been reported to show very low rates of absorption and elimination in European eel [24] and short depletion times (48 h–120 h) from muscle of rainbow trout [25, 26]. More kinetics depletion of this drug was also published also for cold-water fishes including the rainbow trout *Oncorhynchus mykiss* [27], the European eel *Anquilla anguilla* [24, 16, 28], the common carp *Cyprinus carpio* [16], the Atlantic halibut *Hippoglossus hippoglossus* [29–31], in cod *Gadus morhua* and goldsinny wrasse *Ctenolabrus rupestris* [32], the turbot *Scophthalmus maximus* [31] and the channel catfish *Ictalurus punctatus* [7].

Some investigations have been conducted on the sea bass *Dicentrarchus labrax*, a very similar farmed marine fish to sea bream *S. auratus*. The pharmacokinetics of FLU in warm water–farmed sea bass *Dicentrarchus Labrax* was performed without bioavailability data or MICs [10]. The absorption half–life in sea bass following a single intravascular injection of 10 mg/kg on fish held at 18°C was found to be relatively rapid 1.05 h and the elimination half–life of the drug was calculated as 10.71 h, an indication of a fast elimination from plasma. This value is shorter from the same fish kept at 3°C [27]. For the same specie the investigation Luzzana et al. [33] revealed a rapid depletion of FLU from muscle.

Very low levels of 20.6 µg/kg were identified in muscle 12 h following a five day medication period with 12 mg/kg body weight/day at temperature between 21°C and 25.3°C. Very low levels were found in muscle of European sea bass (20.6 ng/g) 12 h after the end of a 5 days standard treatment by [33]. Regarding sea bream, the fish of interest in our research work, previous studies had been conducted under different conditions. The investigation carried out by Malvisi et al [13] revealed a rapid depletion of FLU from muscle. The FLU residues were 10.8±0.5 ng/g in muscle sampled 36 h after the end of a 5 day standard treatment, and no longer detectable (48 h after the end of medication) with a quantitation limit of 10 ng/g.

In the current study FLU was rapidly distributed in the sea bream fish body. The highest concentrations of FLU recorded in liver, red and white muscle were respectively 16.77±6.39 µg/kg, 9.73±5.17 µg/kg and 21.57±24.25 µg/kg at half hour. For the brain, blood, heart, and kidney the highest recorded concentrations were 7.49±0.91 µg/kg, 9.45±1.31 µg/kg, 12.35±2.29 µg/kg and 7.24±0.856 µg/kg 1 h after dosing. The maximum dispersion is recorded for the intestine and spleen at 2 h after dosing and the highest concentrations were respectively, 217±30.15 µg/kg and 57.61±8.4 µg/kg. A fluctuation of the drug was obtained in bile through time and the highest concentration was 749±137.25 µg/kg at 48 h after dosing. The concentration of FLU equivalents in all tissues and organs decreased rapidly after the extensive distribution. At 48 h all the concentrations recorded were below the MRL. The flumequine concentration declined from the fish organs beyond 48 h and was at trace level by the 72nd h. Bile was the main organ retaining the drug, it is thus a good reservoir for FLU retention and confirms that the biliary route is an important excretory pathway in sea bream.

The tissue distribution and residue depletion of FLU after an oral administration have been investigated elsewhere [13] in gilthead sea bream, (*S. aurata L*). The tissue distribution and depletion times of FLU were evaluated in sea bream *S. auratus*, reared under field conditions at 25°C–28°C, after in-feed administration for 5 days at 12 mg/kg body weight/day. The highest concentrations were recorded in vertebrae and skin collected 24 h after treatment (355 ±132.0 ng/g and 317.0 ± 103 ng/g, respectively), while the concentrations in muscles were 68.7±49.6 ng/g. The FLU levels disappeared from muscle 240 h after treatment and showed a longer depletion rate from skin and vertebrae that thus behaved as reservoirs.

The investigation carried out by [11] revealed that FLU concentrations in the muscle plus skin of gilthead sea bream were far below the LOD beyond 168 h after dosing at 18°C and 24°C at 35 mg/kg/day for five days. The results of this depletion study showed that FLU residues are eliminated rapidly from muscle plus skin tissue with elimination half–lives of 22.14 h and 21.43 h at 18°C and 24°C, respectively. Also, withdrawal periods for the MRL of 600 µg/kg FLU in muscle plus skin in natural proportion at 95% tolerance limit were 106 h and 75.84 h at 18°C and 24°C, respectively.

In a similar depletion study in gilthead sea bream (60g–80g) by [13] and held under field conditions at 25°C–28°C and after administration of 12 mg/kg body weight/day in feed for five consecutive days, found lower FLU concentrations in muscle (68.7±49.6 µg/kg), (without skin) and higher in vertebra and skin (355±132.0 µg/kg and 317.0±103 µg/kg, respectively) 24 h after dosing. The FLU levels disappeared from muscle beyond 192 h, but more slowly eliminated from skin and vertebra. These data is support the results obtained in our work. Through the work done elsewhere [26] high level is reported in muscle of rainbow trout of 5,690 µg/kg at 01 day after 05 days of treatment with 12 mg/kg body weight/day at 13.7±0.3°C.

Compared to rainbow trout, the concentrations of FLU in muscle of sea bream were approximately 80 times lower after the same scheduled treatment [26]. In the present study we did not analyse for metabolites. Several studies [3, 34–36] confirm that FLU can be both oxidized and conjugated with glucuronic acid in mammals. In fish the metabolism of FLU appears to be slow, and with the exception of bile only very low levels of metabolites have been detected [6, 30, 37], indicating that the total FLU residues accounted for by means of the applied radioactive tracer techniques consist mainly of the parent compound.

4. CONCLUSION

In conclusion, the present study showed that the pharmacokinetic profile of FLU in sea bream was in accordance with previous reports of marine warm water farmed fishes. However, the drug seems to be an excellent treatment candidate for sea bream because both the distribution and elimination of the drug were found to be rapid. Further studies on tissue distribution and residue depletion of flumequine would provide useful additional data to obtain a thorough understanding of its kinetic profile in Mediterranean fishes.

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ANNEX I.

REPORT ON THE FLU STANDARDS USED IN THE CURRENT RESEARCH

I.1. FLU STANDARDS AND REAGENTS

I.1.1. i-Stable FLU:

Grade: PESTANAL®, analytical standard
Purity: 99.7%.

I.1.2. Ii - Labeled FLU

Compound name: FLU, [2–^{14}C].
Specific Activity: 30.530 mCi/mmol.

The compound was custom synthesized for the FDA by NEN Life Sciences Products (now Perkin–Elmer) in 1999. The radiochemical purity was 97.8% by HPLC in the year 2000.

I.2. REAGENTS

MeCN (LC grade sigma); H_2O (Ultrapure) and MeOH (LC grade Merck).

I.3. APPARATUS

(a) LC System: Knauer system (Germany), 5,000 system controller, S1000 pumps, manual injector, Jetstream oven, UV monitor, Clarity Chrom chromatographic data analyser.

(b) LC column: Supelcosil ODS LC column (150 mm × 3 mm, 3 μm).

(c) LC operating conditions:

Mobile phase: H_2O:MeCN (40/60,v/v), Isocratic; mobile phase flow rate 0.7 mL/min; UV monitor settings, wavelength 324 nm, injection volume 50 μL. Mobile phase: H_2O:MeCN (40/60, v/v).

I.4. PREPARATION OF FLU STANDARDS

Stock solution of both FLU standards of 100 ppm was prepared by dissolution into a 50 mL glass volumetric flask with MeOH. Intermediate standard solutions of 10 ppm were obtained by dilution of the stock solution in MeOH. The LC standard solutions at the following concentrations 300 μg/mL, 600 μg/mL and 1200 μg/mL are obtained by dilution of the intermediate standard solutions in the mobile phase.

TABLE I-1. RADIOACTIVITY VERSUS CONCENTRATION OBTAINED FOR PURITY TEST OF FLU

Concentration	600 ppb	1200 ppb
Mean of three successive measurements of the collected peak of ^{14}C–FLU (first run)	7480 dpm	14718 dpm
Mean of three successive measurements of the collected peak of ^{14}C–FLU (second run)	7410 dpm	14748 dpm
Mean of three successive measurements of the collected peak of ^{14}C–FLU (third run)	7198 dpm	14719 dpm
Mean of the three means of counts obtained from the three runs	7362 dpm	14728 dpm
Purity (%)	94.74	94.75

TABLE I–2. MEAN CONCENTRATION PROFILES OF FLUM EQUIVALENTS IN DIFFERENT TISSUES OF SEA BREAM (N = 3) AFTER A SINGLE 12 mg/kg DOSE ADMINISTERED IP

	Bile		Blood		Brain		Heart		Intestine		Kidney		Liver		Red Muscle		Spleen		White Muscle	
	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean	STD	Mean		Mean	STD
0.5	139.55	78.84	8.11	1.05	6.76	1.44	12.35	2.29	187.72	104.89	4.91	3.56	16.77	6.39	9.73	5.17	13.15	10.26	21.57	24.25
1	92.24	-	9.45	1.31	7.49	0.91	10.48	2.01	149.82	46.27	7.24	8.56	12.43	1.58	7.65	0.87	13.52	7.24	5.65	0.81
2	271.06	169.43	5.18	1.18	5.23	0.68	7.30	2.21	216.70	30.15	5.43	3.45	13.55	2.73	5.92	0.23	57.61	84.35	6.65	0.67
4	429.45	49.86	3.09	1.80	1.87	0.58	2.71	0.43	147.89	27.41	5.37	4.74	4.97	0.88	2.37	0.58	6.70	6.11	2.27	0.28
7	469.09	325.21	0.90	0.28	0.88	0.59	1.08	0.39	67.09	67.86	2.95	0.99	2.17	0.98	0.75	0.36	1.59	0.98	0.76	0.25
24	313.51	179.79	0.82	0.91	0.06	0.04	0.18	0.07	4.39	4.81	0.21	0.06	1.52	1.13	0.09	0.07	0.26	0.28	0.03	0.01
48	748.79	137.25	0.10	0.04	0.05	0.04	0.12	0.05	2.38	1.61	0.14	0.13	0.47	0.42	0.06	0.03	0.19	0.16	0.05	0.04
72	237.62	88.07	0.18	0.07	0.05	0.02	0.22	0.17	3.10	1.35	0.10	0.01	1.76	2.07	0.02	0.00	1.00	0.51	0.01	0.00
96	185.86	170.76	0.26	0.32	0.05	0.04	2.40	4.00	0.90	1.16	0.39	0.37	0.32	0.11	0.02	0.01	0.02	0.01	0.02	0.01
144	57.18	47.71	0.01	0.00	0.02	0.02	0.04	0.03	0.39	0.24	0.12	0.11	0.13	0.04	0.03	0.01	0.11	0.10	0.01	0.00
168	79.43	39.89	1.27	2.11	0.03	0.02	0.07	0.03	1.72	2.41	0.20	0.22	0.52	0.67	0.03	0.01	2.42	3.28	0.01	0.00

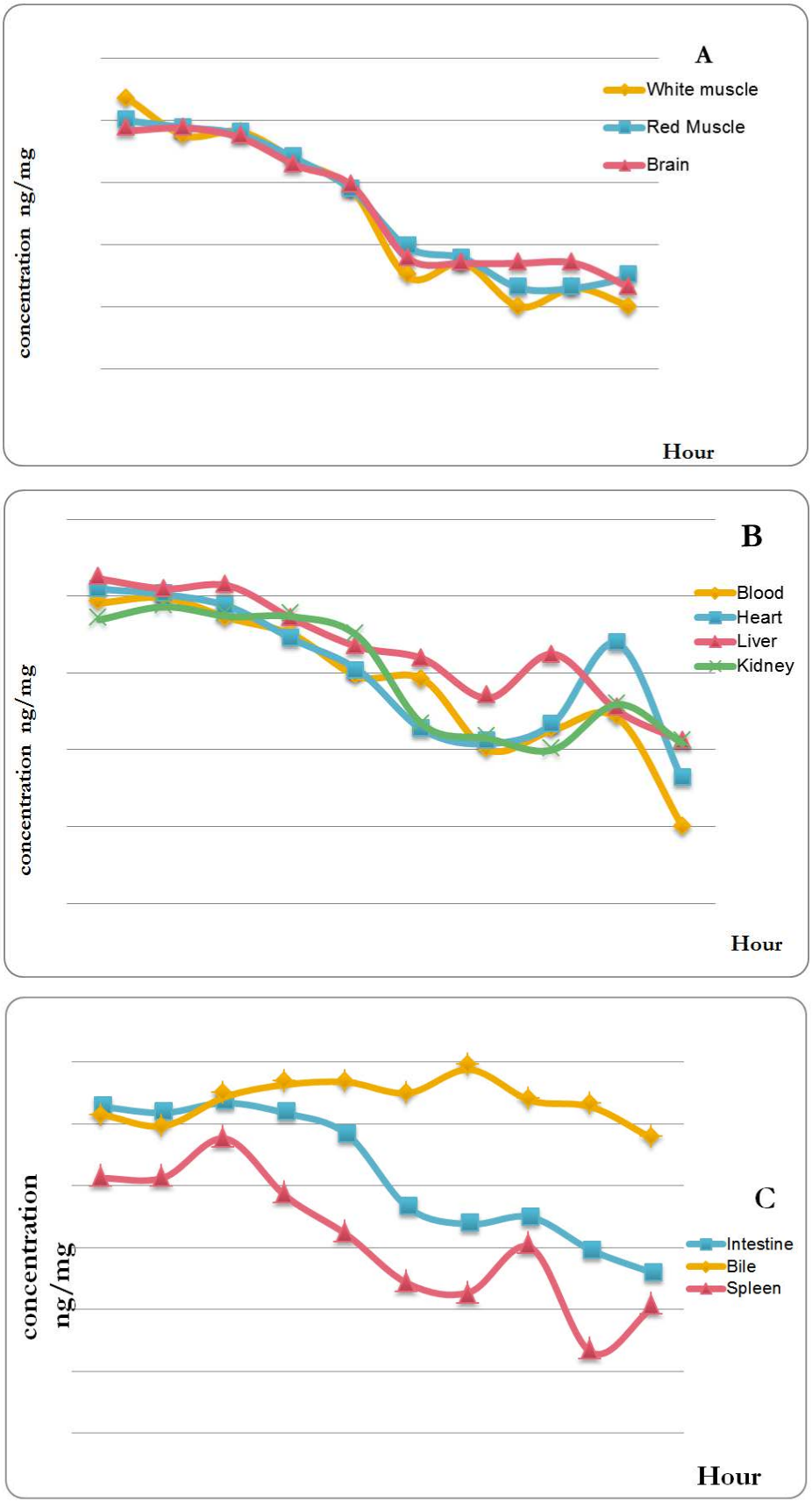

FIG. I-1. Variation of mean concentration of FLU equivalents in several tissues of sea bream through time.

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CONFIRMATORY METHOD FOR THE DETECTION OF AMINOGLYCOSIDES IN MUSCLE, LIVER AND KIDNEY BY LIQUID CHROMATOGRAPHY–ELECTROSPRAY TANDEM MASS SPECTROMETRY

LIU, G., ZHU, Z.,

Chemical Analysis and Physical Testing Center, Shenzhen Center for Disease Control and Prevention, Shenzhen, P.R. China.

Abstract

A quantitative and confirmatory method has been developed for simultaneous determination of 16 aminoglycoside (AG) residues in porcine tissues (muscle, liver and kidney) by liquid chromatography tandem mass spectrometry (LC–MS/MS). The analytes were extracted from different matrices with aqueous trichloroacetic acid solution (5%, w/v) followed by solid phase extraction (SPE) clean–up under optimized conditions. Due to the different pKa values of the compounds, two consecutive SPE steps using Oasis HLB cartridges were used to purify all 16 AGs from sample extracts, with 10 AGs quantitatively retained on Oasis HLB cartridges at pH<1 and the other 6 AGs retained at pH 8.5. The analytes were separated on a reversed-phase C18 column and eluted with water and acetonitrile (MeCN) containing the ion-pair reagent heptafluorobutyric acid (HFBA). The LC–MS/MS method was validated according to Decision 2002/657/EC. The optimized procedure was successfully used to analyse 100 real porcine tissue samples (60 muscles, 20 livers and 20 kidneys) collected from local markets in southern China, demonstrating that the method is robust and useful for determination of residues of the 16 target AGs in porcine tissue samples.

1. INTRODUCTION

Aminoglycosides are a large class of antibiotics with chemical structures characterized by two or more amino sugars linked by glycosidic bonds to an aminocyclitol component, 2–deoxystreptamine, except for streptomycin and dihydrostreptomycin which have a streptidine instead [1]. Some of the drugs are naturally occurring substances isolated from various Actinobacteria (Actinomycetes) particularly members of the Streptomyces and micromonospora genera, while others are semi–synthetic, produced by chemical modification of the natural molecules [2, 3].

The AGs interfere with bacterial protein synthesis by binding irreversibly to ribosomes, which causes damage to the cell membranes [4]. Many of these drugs are extensively used in human and veterinary medicine to treat a wide range of aerobic bacterial infections (mainly Gram–negative bacteria, but some Gram–positive organisms are susceptible) and some protozoal infections [5, 6]. However, the application of these drugs as veterinary medicines may cause the risk of developing antimicrobial resistance among bacteria, which can consequently weaken the efficacy of these drugs as human medicines. Besides, AGs exhibit potential toxicity against the renal, vestibular and auditory systems [3, 7].

To ensure more effective and controlled use of these drugs in animal husbandry, the European Union (EU), JECFA, USA, China, Japan, and other countries/international organizations have issued strict maximum residue levels for nine AGs in various foodstuff of animal origin [8–10]. Therefore, robust, sensitive and reliable analytical methods are required to determine trace levels of drugs such as AG residues in multiple animal products.

A number of analytical methods for the determination of AGs in various matrices have been reported and well documented in the literature [3, 4]. Chemical methods including thin layer chromatography (TLC), capillary electrophoresis (CE), optical biosensors and resonance Rayleigh scattering (RRS), together with biological methods such as enzyme immunoassay, microbiological assay (ELISA) are mainly utilised as screening methods [4]. Since AGs are

non–volatile, they must be derivatized so they are amenable with gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS).

This however requires lengthy derivatization time at elevated temperatures with silylating agents [11]. Since they lack chromophores and fluorophores, AGs are not amenable to direct ultraviolet (UV) or fluorescence detection (FLD) and therefore pre– or post–column derivatization is often required when using liquid chromatography (LC) analysis, followed by the detection with UV or FLD [5, 12–16]. Such LC–UV or LC–FLD methods generally result in poor reproducibility due to the instability and low yield of the derivatives.

Because of this, LC–MS/MS based methods are becoming the normal approach for the confirmatory determination of AG residues because of their high throughput, sensitivity, selectivity and the capability to generate sufficient structural information for analyte confirmation. However, most early LC–MS/MS methods reported are only able to monitor one or two different AGs [12, 17–22]. More recently, more sophisticated sample clean–up and chromatographic separation techniques were introduced and applied in methods capable of determining more AGs within a single analytical method [1, 2, 6, 23–28].

The difficulties in multi–residue LC–MS/MS analysis of AGs in animal products are related to the extraction, clean–up, separation and detection procedures. AGs are highly polar, extremely hydrophilic compounds due to the many amino and hydroxyl groups in their chemical structures, so they are not adequately retained on conventional reversed–phase columns. Thus, many analytical methods use ion–pair reagents such as HFBA [2, 6, 17–20, 23–27] and pentafluoropropionic acid (PFPA) [22] to improve the retention and separation of AGs by reverse–phase LC. Hydrophilic interaction chromatography (HILIC)–MS/MS has also been applied to simultaneously determine various AGs [1, 28]. In general, the currently preferred detection technique for the multi–residue analysis of AGs is LC–MS/MS with electrospray ionization in positive mode [1, 2, 6, 17–28].

Another critical challenge with simultaneous determination of AGs residues in complicated animal matrices is the extraction and clean–up procedure. Matrices such as muscle, liver and kidney contain many interfering substances that need to be selectively removed. Moreover, the AGs are water soluble, highly polar, acid and base resistant compounds which are not extensively bound to proteins. Therefore, the extraction of AGs from complicated food matrices has generally been based on aqueous methods, often using organic solvents (e.g. MeCN [29] or strong acids (e.g. trichloroacetic acid [2, 12, 16, 19, 21–26, 28] or perchloric acid [13, 15]) to precipitate the proteins. Cleaned up has been reported by a variety of methods, including SPE [2, 12–14, 16, 18–20, 22, 24–26, 28], on–line SPE [23], matrix solid–phase dispersion extraction [6] and pressurised liquid extraction [27]. However, most of these purification procedures are suitable for only a few AGs.

A multi–residue method for 11 AGs in meat and liver by LC–MS/MS was reported by Kaufmann et al [24]. The clean–up procedure was quite time consuming, involving 3 steps; anion–exchange, HLB SPE and weak cation–exchange SPE. Bogialli et al [6] proposed matrix solid–phase dispersion with heated water to extract and clean–up 9 AGs in bovine whole milk, which needed special homemade extraction apparatus [6]. Zhu et al [29] developed an LC–ESI–MS/MS method for the simultaneous determination of 13 AGs in muscle, liver, kidney, honey and milk, with sample clean-up by two-consecutive SPE steps using two HLB cartridges at pH <1 and pH 8.5 [26]. Kaufmann et al [2] carried out a single–step strong cation–exchange SPE procedure to clean up 13 AGs in pork muscle, fish, veal liver and kidney [2]. Tao et al [28] applied auto SPE using weak cation–exchange carboxylic

acid cartridges for sample clean–up and utilized a specialized column for AGs to achieve chromatographic separation for the determination of 15 AGs. However, gentamicin C2 and gentamicin C1a, two important isomers of gentamicin, were not included [28].

Therefore, the main objective of this study was to develop a multi–residue LC–ESI–MS/MS method for the simultaneous determination and quantification of 16 AGs including apramycin (APRA), streptomycin (STREP), dihydrostreptomycin DISTREP, amikacin (AMIK), spectinomycin (SPEC), kanamycin (KANA), neomycin (NEO), paromomycin (PARO), tobramycin (TOBRA), gentamicin (GENT, consisting of isomers C1, C1a and C2), hygromycin B (HYGRO), netilmicin (NETIL), sisomicin (SISO), and micronomicinin (MICRONO) in porcine tissue (muscle, liver and kidney) samples. Samples in 5% trichloroacetic acid (TCA) were extracted by homogenization followed by solid phase extraction (SPE) clean–up before analysis. Experimental conditions for sample preparation and instrumental analysis were optimized to achieve best performance. The optimized method was then applied to determine AGs in porcine tissue samples collected from local markets in southern China.

2. MATERIALS AND METHODS

2.1. CHEMICALS AND REAGENTS

All reagents were of analytical reagent quality or better. APRA, AMIK, SPEC, KANA, NEO, PARO, STREP, DISTREP, TOBRA, and GENT (consisting of C1a, GENT C2, GENT C1) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany), while HYGRO aqueous solution (54 mg/mL), NETIL, SISO, and MICRONO were purchased from Sigma–Aldrich (St. Louis, MO, USA), European pharmacopoeia (Strasbourg, France), Toronto Research Chemicals Inc. (Toronto, Canada) and International laboratory USA (South San Francisco, CA, USA), respectively. HFBA (>99.5%) was supplied by Sigma–Aldrich (USA). Acetonitrile (MeCN), methanol (MeOH), glacial acetic acid and n-hexane were obtained from Merck (Germany). Analytical grade TCA, Sodium hydroxide (NaOH) and Hydrochloric acid (HCl) were supplied by Guangzhou Chemical Company (Guangzhou, China). Ultrapure water (18.2 MΩ.cm) was generated by a Milli–Q® Advantage A10 water–purification system (Millipore, France). The cartridges used for solid phase extraction (SPE) were Oasis HLB cartridges (3 cc/60 mg) from Waters (Milford, MA, USA). Filter membranes (0.45 µm) provided by Jinteng laboratory facilities Co. Ltd (Jinteng, Tianjin, China) were used to filter the sample solutions before injection into LC–ESI–MS/MS system. Aminoglycoside standards in the purity range of 90% to 99% were obtained from Dr. Ehrenstorfer Gmbh and Toronto Research Chemicals Inc., Canada. Micronomicin sulphate (International laboratory, USA) and HYGRO B (Sigma–Aldrich, USA) had lower purity levels ~ 60%.

2.2. STANDARD SOLUTIONS

Standard solutions were prepared using MeCN:H_2O:acetic acid (20:78:2, v/v/v). Individual stock solutions of all 16 AGs (100 µg/mL, except GENTA which was 80 µg/mL) were prepared in diluted solution and kept stable for 6 months (m) when stored in plastic tube at 2–4ºC. A tuning solution of each analyte (10 µg/mL) was prepared by diluting individual stock solutions with diluting solution. A working standard mixture used to fortify three samples, was prepared by diluting individual stock solution of 16 AGs with diluted solution in appropriate concentrations to a final volume of 50 mL, (Tables 1–3). When a lower

fortification mixture was needed, an extra dilution of the AGs was prepared. These solutions were stored in plastic tubes at 2ºC–4ºC and remained stable for up to 1 m.

TABLE 1. PREPARATION OF WORKING STANDARD MIXTURE OF TARGET AGs FOR PORCINE MUSCLE.

Compound	MRL/Suggesting level for no MRL (µg/kg)	Concentration of individual solution (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)
STREP	500	100	6.25	12.5
DISTREP	500	100	6.25	12.5
NEO	500	100	6.25	12.5
PARO	500	100	6.25	12.5
KANA	40	100	0.50	1
AMIK	/ (100)	100	1.25	2.5
TOBRA	/ (50)	100	0.625	1.25
SPEC	100	100	1.25	2.5
APRA	60	100	0.75	1.5
GENT C1	50			1.164
GENT C2	50	80 （total GENT）	2.50	1.984
GENT C1a	50			0.852
HYGRO	/ (500)	100	6.25	12.5
NETIL	/ (50)	100	0.625	1.25
SISO	/ (50)	100	0.625	1.25
MICRONO	/ (50)	100	0.625	1.25

TABLE 2. PREPARATION OF WORKING STANDARD MIXTURE OF TARGET AG_S FOR PORCINE LIVER.

Compound	MRL/Suggesting level for no MRL (µg/kg)	Concentration of individual solution (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)
STREP	500	100	5	10
DISTREP	500	100	5	10
NEO	500	100	5	10
PARO	1500	100	15	30
KANA	40	100	0.4	0.8
AMIK	/ (100)	100	1	2
TOBRA	/ (50)	100	0.5	1
SPEC	100	100	1	2
APRA	100	100	0.6	1.2
GENT C1	100			1.8624
GENT C2	100	80 (total GENT)	4	3.1744
GENT C1a	100			1.3632
HYGRO	/ (500)	100	5	10
NETIL	/ (50)	100	0.5	1
SISO	/ (50)	100	0.5	1
MICRONO	/ (50)	100	0.5	1

TABLE 3. PREPARATION OF WORKING STANDARD MIXTURE OF TARGET AGs FOR PORCINE KIDNEY.

Compound	MRL/Suggesting level for no MRL (µg/kg)	Concentration of individual solution (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)
STREP	1000	100	2.5	5
DISTREP	1000	100	2.5	5
NEO	5000	100	12.5	25
PARO	1500	100	3.75	7.5
KANA	40	100	0.1	0.2
AMIK	/ (100)	100	0.25	0.5
TOBRA	/ (50)	100	0.125	0.25
SPEC	500	100	1.25	2.5
APRA	100	100	0.25	0.5

Compound	MRL/Suggesting level for no MRL (μg/kg)	Concentration of individual solution (mg/L)	Aliquot taken (mL)	Concentration of each compound in resulting mixture (mg/L)
GENT C1	200			0.9312
GENT C2	200	80 (total GENT)	2	1.5872
GENT C1a	200			0.6816
HYGRO	/ (500)	100	1.25	2.5
NETIL	/ (50)	100	0.125	0.25
SISO	/ (50)	100	0.125	0.25
MICRONO	/ (50)	100	0.125	0.25

2.3. SAMPLES

Porcine tissue (muscle, liver and kidney) samples collected from Shenzhen local markets were homogenized to paste by a high speed blender, stored at -20ºC ± 5ºC in plastic containers, and analysed within 3 m. Samples would be thawed to cold storage immediately after sub-sampling and analysed soon thereafter or stored again at -20ºC ± 5ºC. Care was taken while handling samples to prevent accidental contamination or loss of target compounds.

2.4. SAMPLE PREPARATION

2.4.1. Extraction

Five grams tissue sample aliquot was weighed into polypropylene centrifuge tube and fortified with the working standard solution at appropriate concentrations and 10 mL of 5% TCA (w/v) added to the centrifuge tube. The mixture was homogenized thoroughly at 10 000 rpm for 1 min and then centrifuged at 8000 rpm for 5 min. This was repeated with 10 mL of 5% TCA, and the TCA supernatants combined in separate centrifuge tube and 5 mL 0.2M HFBA and 5 mL n–hexane were added to these extracts. After vibration–mixing at 360 rpm for 30 min and additional centrifugation at 8000 rpm for 5 min, the upper n-hexane phase was removed with the upper layer extracts ready for the clean–up procedure.

2.4.2. SPE clean–up and concentration

A HLB cartridge was preconditioned with 3 mL MeOH, 3 mL H_2O, and 3 mL 0.2M HFBA by gravity. Then 5 mL of the previous upper layer extract was transferred onto the cartridge at a flowrate of 1 mL/min. After penetration, the cartridge was dried by a vacuum pump for 5 min. All the effluent was collected into another tube and adjusted to pH 8.5±0.2 with 100 g/L NaOH (about 9 drops) and 0.2 moL/L HCl. Another HLB cartridge was preconditioned with 3 mL MeOH, 3 mL H_2O, 3 mL 0.2M HFBA and 3 mL pH 8.5 NaOH solution by gravity. Then, the pH 8.5±0.2 effluent was loaded onto the column at 1 mL/min. After the sample was absorbed, two HLB cartridges were hyphenated with vacuum joints. The two hyphenated cartridges were rinsed with 5 mL H_2O, then dried at less than 15 mmHg for 10 min. The AG residues were finally eluted with 6 mL MeCN/0.15M HFBA (8:2, v/v) and the elution evaporated to 0.3 mL under a gentle steam of nitrogen at 40ºC. Finally, the residue was reconstituted to 1 mL with 20mM HFBA. The resulting solution (filtered through a 0.45 μm membrane first if turbid) was transferred into an LC autosampler vial, and analysed by LC–ESI–MS/MS.

2.5. LC–MS/MS ANALYSIS

2.5.1. LC condition

Liquid chromatography was performed using an Agilent 1100 HPLC system equipped with an automatic degasser, a quaternary pump and an autosampler. Chromatographic separation was carried out using an Atlantis® dC18 column (150 mm×2.1 mm, particle size 5 µm) at 30ºC. The mobile phase flow–rate was maintained at 0.4 mL/min and the injection volume was 30 µL. The mobile phase C was MeCN:H_2O (5:95, v/v) containing 20mM HFBA, mobile phase D was MeCN:H_2O (50:50, v/v) containing 20mM HFBA, and mobile phase A was MeCN containing 20mM HFBA. The gradient elution program used is summarized in Table 4.

TABLE 4. HPLC GRADIENT ELUTION PROGRAMME.

Time (min)	C (%)	D (%)	A (%)
0.00	90	10	0
1.00	90	10	0
5.00	50	50	0
8.00	50	50	0
11.00	35	65	0
11.10	0	5	95
13.90	0	5	95
14.00	90	10	0
18.00	90	10	0

2.5.2. MS/MS parameters

The HPLC system was connected to an API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems/MDS Sciex, Toronto, Canada), equipped with a turbo ionspray source and a syringe pump. The electrospray ionization mode (positive) was adopted. Optimization of mass condition was achieved by infusing each compound separately at a flow–rate of 10 µL/min. Detection was operated in the multiple reaction monitoring (MRM) mode and the acquisition of two transitions made it possible to obtain at least three IPs as required by the 2002/657/EC guidelines [30]. The higher intensity transition was selected for quantitative purpose and the resolution was set at unit (0.7 U).

The instrument control and data acquisition were carried out by the analyst 1.4.1 software. Mass parameters for each analyte including precursor ion (Q1), product ion (Q3), declustering potential (DP), entrance potential (EP), cell exit potential (CXP), collision energy (CE) are summarized in Table 5. The focusing potential (FP) and dwelling time (DT) for all analytes were 350 V and 40 msec, respectively. The MS ion source parameters including nebulizer gas (NEB), curtain gas (CUR), collision gas (CAD), ion spray voltage (IS) and ion source temperature (TEM) was 12 psi, 8 psi, 6 L/min, 3,500 V and 500ºC, respectively.

TABLE 5. HPLC GRADIENT ELUTION PROGRAMME.

Compound	MW	Precursor ion *(m/z)*	Product ion *(m/z)*	DP (V)	EP (V)	CXP (V)	CE (V)
APRA	539.6	540.4	*378.3 217.2	105	4.2	23 13	25 40
AMIK	585.6	586.3	*425.2 264.1	90	4.2	27 17	29 38
SPEC	332.3	351.3	*333.2 98.2	60	10.0	23 6	26 44
NEO	614.6	615.4	*161.2 293.0	155	4.3	10 17	44 36
TOBRA	467.5	468.3	*163.2 324.1	65	10.0	10 19	36 23

Compound	MW	Precursor ion *(m/z)*	Product ion *(m/z)*	DP (V)	EP (V)	CXP (V)	CE (V)
GENT C1a	449.5	450.3	*160.1 322.1	85	5.0	9 20	34 20
GENT C2	463.6	464.3	*322.1 160.1	85	5.0	20 9	20 34
GENT C1	477.6	478.3	*157.2 322.2	100	4.5	10 20	30 21
KANA	484.5	485.3	*163.2 324.1	80	4.3	9 19	39 25
HYGRO	527.5	528.2	*177.2 352.2	95	10.0	10 20	44 35
DISTREP	583.6	584.2	*263.1 246.2	145	9.5	14 14	46 56
PARO	615.6	616.3	*163.2 293.0	135	9.0	11 17	52 35
STREP	581.6	600.3	*582.2 263.1	125	4.5	34 16	26 52
NETIL	475.6	476.4	*299.5 191.4	65	7.2	21 11	31 36
SISO	447.5	448.5	*322.4 271.5	50	7.0	20 19	20 27
MICRONO	463.6	464.6	*322.4 160.3	90	7.6	22 15	21 33

* Selected as quantitative ion

2.6. METHOD VALIDATION

The LC–MS/MS method was validated according to 2002/657/EC guidelines [30] for confirmation analysis. According to the criteria, the performance characteristics including recovery, repeatability, decision limit (CCα), detection capability (CCβ), calibration curves, stability, specificity together with LOD and limit of quantification (LOQ) in porcine tissue (muscle, liver and kidney) matrices were evaluated.

2.6.1. Calibration curves

The matrix-match calibration standard curves were prepared by fortification at 0, 0.25, 0.5, 1.0, 2.5, 5.0 times MRL (muscle), 0, 0.2, 0.4, 0.8, 2.0, 4.0 times MRL (liver), and 0, 0.2, 0.5, 1.0, 2.5, 5.0 times MRL (kidney). Negative control samples were taken through the entire extraction procedure and spiked with the analytes to be determined at the end of the extraction immediately before (or very close to) analysis. The calibration curves were constructed using a peak area from six concentration levels versus the concentration of analytes according to linear regression. Thus, there were always 16 different matrix–match calibration curves for each test sample.

2.6.2. LOD and LOQ

The LOD and LOQ were calculated in the spiked negative samples (porcine muscle, liver and kidney; at least in six aliquots) as three and ten times of S/N ratio for each compound, respectively. The calculated LOD and LOQ for all AGs were summarized in Table 6.

TABLE 6. CALCULATED LOD AND LOQ FOR THE TARGET ANALYTES IN THREE MATRICES (μg/kg)

Compound	Muscle			Liver			Kidney		
	MRL	LOD	LOQ	MRL	LOD	LOQ	MRL	LOD	LOQ
APRA	60	6.5	21	60	5	16	100	6	20
AMIK	/	7	24	/	6	20	/	8	25
SPEC	100	13.5	45	100	21	65	500	12	39
NEO	500	16	53	500	19	63	5000	38	125
TOBRA	/	4	13	/	3	10	/	4.5	15

Compound	Muscle			Liver			Kidney		
	MRL	LOD	LOQ	MRL	LOD	LOQ	MRL	LOD	LOQ
GENT C1a	50	3.5	12	100	2.5	8	200	3	10
GENT C2	50	4	13	100	4	13	200	4	13
GENT C1	50	3	10	100	2.5	8	200	4	13
KANA	40	4.5	15	40	3.7	12	40	6	20
HYGRO	0	62	200	0	75	250	0	45	150
DISTREP	500	8	27	500	12	40	1000	35	120
PARA	500	16.5	54	1500	10	32	/	13	42
STREP	500	21	68	500	29	96	1000	12	40
NETIL	/	3	10	/	8	25	/	4.5	15
SISO	/	5.5	18	/	3.5	11	/	5	16
MICRONO	/	3	10	/	3	10	/	3	10

2.6.3. Recovery and precision

Porcine muscle, liver and kidney known to be compliant served as blank matrices. Recoveries and precisions (intra–day, inter–day) were calculated from the determination of 7 aliquots each sample fortified at three levels [0.5, 1.0, 1.5 times MRL or 0.5, 1.0, and 1.5 times a suggested level (probably MRPL) for no MRL substances]. The analyses were completed by the same operator in triplicate. The recoveries were calculated by the measured content/the fortified level×100 and precision was expressed as the RSD.

2.6.4. The CCα and CCβ

The CCα is the lowest concentration at which a method can discriminate with a statistical certainty of 1-α that the analyte is present. For all 16 AGs, CCα was established by the following: 21 blank matrices of porcine muscle, liver and kidney samples spiked at the MRL (or suggested level) were analysed. The average measured concentration at the MRL (suggested level plus 1.64 times the corresponding standard deviation (SD) is defined as CCα (α=5%). The CCβ is the concentration at which the method is able to detect truly contaminated samples with a statistical certainty of 1-β. For all 16 AGs, CCβ was calculated by analysing 21 porcine muscle, liver, kidney spiked with the analytes at CCα and then the CCα value plus 1.64 times the corresponding SD is equal to CCβ (β=5%).

2.6.5. Specificity

The specificity of the method was demonstrated by testing all matrices available in our research. In total 20 muscle, 20 kidney, 20 liver (n=60) samples were studied. These were fortified with identical concentration (40~160 μg/kg for 16 AGs). The results were evaluated by the presence/absence of interfering substances around the AGs' retention time.

2.6.6. Stability

Stability of the analytes was evaluated in a simplified version involving selected key parameters/factors, namely: (1) evaluating the stability in different solutions, where all AGs (100 μg/mL) were dissolved in MeCN, MeCN:H_2O (50:50, v/v), or MeCN:H_2O:acetic acid (20:78:2, v/v/v); (2) assessing the stability in three storage conditions, where the stock solutions (100 μg/mL) and lower concentration (for instance 100 ng/mL) were stored at room temperature (about 20ºC) 4ºC, or -20ºC; (3) placing the stock solutions in glass or plastic tube. The measured values were compared to those of freshly prepared standard solutions.

3. RESULTS AND DISCUSSION

3.1. METHOD DEVELOPMENT

3.1.1. Optimization of sample preparation procedure

The sample pre–treatment procedures were studied step by step based on porcine muscle matrix, including extraction solvent, centrifuge conditions and solid–phase extraction conditions. An IKA® T25 digital Ultra–Turrax® high speed blender with IKA® works S25N–25F dispersing was used for sample homogenization. Centrifugation was done at 8000 rpm and 5ºC since the centrifugation time increased with temperature (10ºC and 20ºC). Since pork muscle contains a lot of fat, 5 mL of n–hexane was used to remove any remaining fat in the HFBA acidified TCA extracts before loading onto HLB cartridges. This was compared to when no n–hexane was added. Recoveries of the 16 AGs improved with defatting.

The influence of HFBA concentration used for preconditioning of HLB cartridge was investigated by testing HFBA at 0.02 mol/L, 0.05 mol/L, 0.10 mol/L, 0.15 mol/L, 0.20 mol/L, 0.25 mol/L, 0.30 mol/L and 0.40 mol/L. Recovery of most AGs increased with HFBA concentration and achieved a maximum value at 0.20 mol/L beyond which no significant improvement in recovery was seen. Therefore, 0.20 mol/L HFBA was adopted.

Since the 16 AGs have different pKa values, the pH of the solution loaded onto columns influences performance. Hence, HFBA acidified TCA extracts were adjusted to pH 0.5, 1.0, 1.5, 2.0, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, 11.0 in replicate and then loaded on to a single conditioned HLB column to assess the performance under different pH conditions. Optimum recoveries were observed at pH 1.0 for only 10 AGs, while high recoveries for other 6 AGs were found at pH 8.5. After many trials, the 16 AGs could not be retained or eluted on the columns when only one HLB column was applied i.e. the optimized overall performance could be obtained with two coupled HLB columns under different pH conditions. Furthermore recoveries were comparable for the HFBA acidified TCA extracts with no pH and pH 1.0 adjustments. Thus, the 5 mL n–hexane defatted and HFBA acidified TCA extracts were directly loaded on to the first HLB cartridge, the eluent collected and adjusted to pH 8.5 for clean–up on the second HLB cartridge.

Considering that the pH of the effluent from the first column was adjusted to 8.5, the introduction of alkaline solution for preconditioning of the second HLB cartridge was done under different pH conditions. Specifically, the second cartridge was preconditioned with 3 mL each of MeOH, H_2O and NaOH at different pH values. As a result, 3 mL each of MeOH, H_2O, 0.2M HFBA and pH 8.5 NaOH was found appropriate.

The elution procedure of the two HLB cartridges was also investigated by comparing the elution efficiency of different solvents (at different volumes), such as 6 mL MeCN, 6 mL MeOH/0.2M HFBA (8:2, v/v), 3 mL MeOH, 3 mL MeOH/NH_3 (9:1, v/v), 6 mL MeCN/0.2M HFBA (8:2, v/v). Ultimately 6 mL of MeCN/0.2M HFBA (8:2, v/v) showed the best elution efficiency.

Further investigation was done and finally, 6 mL of MeCN/0.15M HFBA（4:1, v/v）showed the best elution efficiency, and was applied for elution of the 16 AGs from the two hyphenated HLB cartridges. Besides, several other kinds of preparation methods for AGs in foods of animal origin using different commercially available SPE cartridges (Varian AccuBOND ODS–C18, Waters Oasis MCX, Waters Oasis WCX, Supelclean™ LC–WCX)

were evaluated and we observed that all the 16 AGs could not be quantitatively retained or eluted on the columns, compared to our method.

3.1.2. Mass spectrometry

Each AG tuning solution was directly injected into the electroionspray source using a syringe pump. Full scan and collision activated dissociation (CAD) tests were operated to set up an appropriate MRM method. Protonated molecular ions $[M+H]^+$ were predominant for the AGs in positive ESI mode and were thus selected as precursor ions except for SPEC and STREP, which produced an intense water adduct $[M+H_2O+H]^+$. For the confirmation of group B substances in foodstuffs, a minimum of three identification points (IPs) is required according to EU criteria and the method fulfilled this requirement with the use of two MRM transitions (one precursor and two product ions) for each compound, which count for four IPs. The characteristic fragment ions of all 16 AGs were obtained in product ion scan mode.

The LC gradient program was 18 min with good peak shape and proper separation. All 16 AGs also showed sufficient data points in both mixed standard solutions and fortified samples. The chromatograms are shown in figure 1.

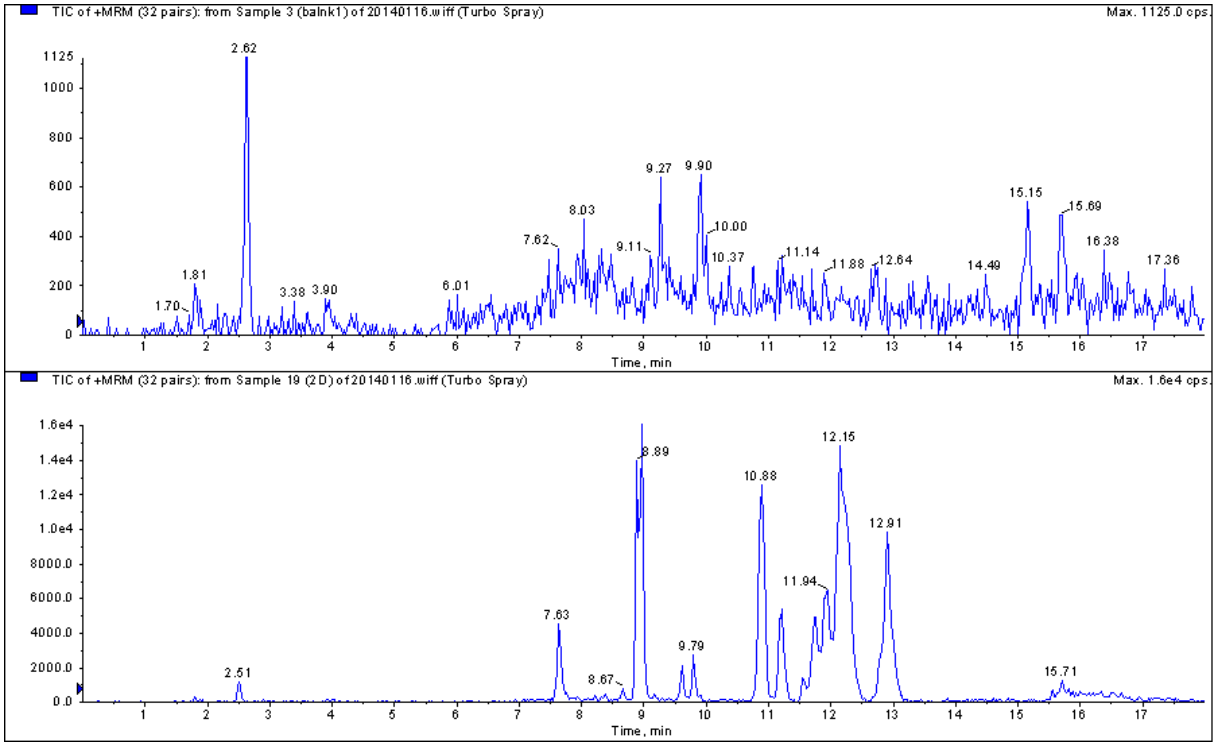

FIG.1. Total Ion Chromatograms of a blank (upper) and spiked (lower, 1.0×MRL ~ 40~500 µg/kg of the 16 AGs) pork sample.

3.2. METHOD VALIDATION

All performance data of this procedure are summarized in Table 7. The AG peak areas and concentrations were fitted to linear equations in the range of 0–5 times MRL or suggesting levels. For each compound, three matrix matched calibration curves were prepared and no significant differences were observed. The correlation coefficients for each calibration curve

were higher than 0.99. The recoveries ranged from 51% to 112% with overall precisions of 2.8%–13.3%. The CCα and CCβ values were in the range of 44.7 μg/kg–5,358 μg/kg and 49.0 μg/kg–5795 μg/kg for all analytes, respectively.

After comparing with the background noise of three matrices, the results demonstrated that, there were no interfering peaks that could be detected at the expected retention time window (±2.5%) for these target analytes. The optimum conditions for storage of all analytes were as follow: the stock solutions (100 μg/mL dissolved in MeCN:H_2O:acetic acid (20:78:2, v/v/v) and lower concentration (such as 100 ng/mL) were stable in plastic tube at 4ºC for at least 6 months and for 1 week, respectively.

3.3. METHOD APPLICATION TO REAL SAMPLE ANALYSIS

The method was successfully used to conduct a regional survey for AGs in 100 real porcine tissue samples (60 muscles, 20 livers and 20 kidneys) collected from different cities of Guangdong Province, China and analysed in our laboratory. Only KANA and STREP were simultaneously found in two porcine kidneys at concentration of 991 μg/kg (KANA), 346 μg/kg (STREP) and 29, 114 μg/kg (KANA), 14, 600 μg/kg (STREP), respectively.

3.4. COLLABORATIVE STUDY

The entire LC–MS/MS confirmatory method was tested for reproducibility through a collaborative study that involved first the training of and application of the method by an analyst from Botswana in a period of 2 weeks. To confirm its robustness, the method has also been transferred from China to the Botswana National Veterinary Laboratory.

TABLE 7. THE VALIDATION RESULTS OF 16 AGs IN THREE MATRICES

	SPEC	HYGRO	STREP	DISTREP	AMIK	KANA	APRA	PARO	TOBRA	SISO	GENTC1a	GENTC2	MICRONO	NETIL	GENTC1	NEO
Muscle																
Intra—day RSD (%) n=21	3.9-9.5	4.1-8.7	4.8-10.6	5.2-8.0	3.5-9.6	4.1-9.4	4.3-12.2	3.0-11.2	3.5-8.6	4.1-11.1	3.7-10.1	3.3-9.7	4.4-12.1	4.7-12.6	4.2-11.7	5.2-10.3
Inter—day RSD (%) n=63	5.0-10.4	7.4-10.1	8.2-11.1	5.3-8.2	5.5-8.4	6.4-7.8	5.7-10.5	5.6-8.9	6.4-8.2	5.9-9.5	5.4-8.8	7.0-10.3	4.8-9.3	9.1-12.2	5.6-9.6	5.5-9.2
Recovery (%)	60-85	51-78	64-98	74-107	60-90	66-88	66-101	78-107	76-108	76-107	76-106	76-111	76-110	74-112	76-111	75-102
MRL/suggesting level (μg/kg)	100	/500	500	500	/100	40	60	500	/50	/50	50	50	/50	/50	50	500
CCα (μg/kg)	113.6	549.5	561.7	564.7	105.1	44.7	63.2	551.4	51.9	54.8	55.2	54.9	55.2	56.4	54.0	555.0
CCβ (μg/kg)	125.6	595.8	626.0	627.7	109.9	49.0	65.8	599.9	53.9	60.0	59.9	59.7	60.3	62.6	59.2	612.3
Liver																
Intra—day RSD (%) n=21	3.3-9.1	4.0-7.2	5.4-9.8	4.6-8.5	4.1-7.8	3.2-10.0	4.2-9.8	4.5-9.5	3.4-7.1	3.4-13.3	3.4-10.2	4.6-10.7	3.6-11.3	2.8-11.0	4.2-9.5	4.9-10.3
Inter—day RSD (%) n=63	5.4-9.4	6.2-6.8	8.4-9.7	8.8-10.6	8.8-10.0	7.1-8.6	4.9-10.4	5.5-8.6	5.6-10.7	6.6-10.6	5.5-8.8	7.2-10.4	7.5-11.5	6.7-10.5	7.4-8.9	7.1-10.7
Recovery (%)	59-85	56-72	61-92	69-102	60-87	59-84	66-95	75-100	63-101	69-100	73-110	69-106	70-105	73-109	77-109	71-100
MRL/suggesting level (μg/kg)	100	/500	500	500	/100	40	60	1500	/50	/50	100	100	/50	/50	100	500
CCα (μg/kg)	109.3	541.5	549.8	552.5	108.0	45.9	65.7	1630.8	51.6	54.8	111.6	109.4	57.8	57.7	112.0	555.2
CCβ (μg/kg)	117.6	581.0	609.0	610.9	116.6	50.9	72.5	1758.9	54.6	59.6	125.4	121.3	66.5	66.2	124.9	618.7
Kidney																
Intra—day RSD (%) n=21	4.7-8.4	4.2-6.9	3.3-9.6	3.5-8.0	2.9-8.3	3.8-8.8	4.4-10.1	3.8-8.9	5.0-9.5	3.5-9.5	4.2-10.2	3.5-8.1	3.8-13.8	2.8-9.3	4.2-8.2	4.7-10.2
Inter—day RSD (%) n=63	5.6-10.4	7.0-7.9	8.3-8.6	8.3-10.8	6.1-7.6	9.0-9.7	5.5-8.1	5.6-8.0	6.0-8.7	9.6-10.1	6.6-10.3	5.8-7.3	5.0-9.8	6.6-9.6	7.0-8.6	10.1-11.4
Recovery (%)	60-91	54-77	68-100	70-106	74-100	61-88	76-101	76-103	74-103	68-101	68-103	76-102	69-105	69-98	75-107	67-99
MRL/suggesting level (μg/kg)	500	/500	1000	1000	/100	40	100	1500	/50	/50	200	200	/50	/50	200	5000
CCα (μg/kg)	570.3	559.9	1104.2	1104.9	108.9	45.2	111.0	1640.5	56.9	54.1	227.8	217.5	57.3	55.5	226.3	5357.5
CCβ (μg/kg)	630.7	609.6	1206.2	1221.9	117.3	50.3	123.7	1788.8	63.3	59.4	257.3	237.3	65.1	62.1	252.7	5795.3

Spike levels were 0.5, 1.0 and 1.5 × MRL/suggesting levels

4. CONCLUSION

A method was developed and validated for the quantitative determination of 16 AGs in porcine liver, kidney and muscle. The baseline separation of all 16 AGs was achieved within an 18 min gradient elution program. The method showed good sensitivity, and the performance characteristics of the method comply with EU recommendations. Sufficient IPs were generated for the confirmation of the identity of all 16 target AGs according to EU criteria. The method was successfully applied to determine AGs in porcine tissue samples from local markets in southern China. In terms of overall data and consideration, this method should be an efficient approach for multi–residue analysis of AGs in animal tissues.

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DEVELOPMENT, STANDARDIZATION AND APPLICATION OF A MULTIPARAMETRIC ASSAY, DOT–ELISA–MULTI, TO DETECT ENROFLOXACIN AND CIPROFLOXACIN IN CHICKEN MEAT

NOGUEIRA, G, D.P.,

Universidade Estadual Paulista (UNESP), Faculdade de Medicina Veterinaria; Departamento de Apoio, Producao e Saude Animal, Rua Clovis Pestana, 793, 16050-680, ARACATUBA, SP, BRAZIL

Abstract

A multiparametric assay, Dot–ELISA–Multi for the detection of enrofloxacin (ENR) and ciprofloxacin (CIP) in chicken meat has been investigated and specific factors contributing to successful production of antibodies studied. ENR–KLH stimulated better antibody production than ENR BSA. Repeated inoculation of rabbits was however required. Also an accurate, reproducible and highly sensitive ESI LC–MS/MS method for testing ENR and CIP in five chicken matrices (plasma, muscle, fat/skin, kidney and liver) was validated according to European Community and FDA guidelines, and applied. The detection and quantification limits were found to be low enough to determine ENR and CIP residues in chicken tissues below the permissible maximum recommended residue limits established by the European Medicines Agency. Broiler chickens were successfully used to determine depletion of enrofloxacin in muscle, kidney, liver and plasma.

1. INTRODUCTION

There is need for an analytical method to detect two commonly used antibimicrobial residues in poultry meat outside a laboratory such as a colorimetric multiparametric dot–ELISA. This would involve few steps, be fast and affordable. Brazil's animal industry has grown remarkably over the last 50 years and required use of antibiotics such as quinolones that may result in residues in the animal products. The research was conducted to develop a multiparametric rapid test to detect enrofloxacin and ciprofloxacin residues in the liver, kidney, muscle, fat and skin of chicken.

As the world agribusiness has expanded, research institutes in Brazil have played an important role in the development of new technologies to improve agriculture production [1] Brazil is one of the few countries that has the capability for a significant increase in both plant and animal production [2] due to its large size, a diverse weather conditions, abundant rain, solar energy and because it has 13% of all world fresh water [3].

Since the middle of the last century, the Brazilian animal industry especially aviculture, has developed and thus increasing meat and egg production year after year, which is in turn reflected in lower productive cost and increasing consumption [4] However, huge increase in aviculture would also be associated with increased use of antibiotics [5].

Most of the products used in commercial poultry production are prophylactic, with the specific objective to preserve productivity of the breeding stock, increase feed conversion and consequently weight gain; and controlling clinical or sub–clinical disease development [6].

Maintenance of poultry health is an obligation of poultry farmers, increase productivity and reduce economic loss as well as ensuring animal welfare. While it is therefore obvious that the utilization of antibiotics in poultry production is essential [6], it is very important to respect withdrawal periods established for each product to avoid residue in animal products [7]. For example, following use, it is possible to detect residues of ENR including its metabolite CIP in muscle, kidney, liver, lungs, fat, and skin from chicken. It is thus necessary that a withdrawal period of at least 12 days is observed to decrease such residues to below the established maximum limit of residues (MRLs) for poultry meat [8].

According to the Council Regulation 2377/90 [9], the quantification of MRLs guides antibiotic utilization in animals to ensure consumer safety. The MRL is the maximum drug fraction drug residue legally allowed in raw material or in food products derived from drug treated animal [10]. According to Codex Alimentarius, Food and Drug Administration (FDA/USA) or European Medical Evaluation Agency (EMEA), levels below the MRL pose no harm to consumer health [2].

2. MATERIALS AND METHODS

2.1.ANTIBODY PRODUCTION

2.1.1. Antigen preparation for immunization

As the antibiotics (being small antigens) are not immunogenic enough to stimulate an immune response in rabbit, it was necessary to conjugate the drugs with a protein. CIP (Fluka analytican cod.17849) and ENR (Fluka analytican cod.17850) were conjugated to mariculture keyhole limpet hemocyanin (mcKLH, code and BSA using a commercial conjugation kit from Pierce Biotechnology (Rockford, USA, cod 77601) known as Imject® Immunogen EDC Kit with mcKLH and BSA. This kit contains: Imject® mcKLH (5×2 mg lyophilized), Imject® BSA (5×2 mg lyophilized); Imject® EDC Conjugation Buffer (30 mL), EDC (1–ethyl–3– [3–dimethyllaminopropyl] carboimide hydrochloride, 10×10 mg), D–Salt™ Dextran desalting columns (10×5 mL) and Imject® Purification Buffer Salts (5×5 g).

To conjugate the antibiotic to a carrier protein the presence of functional groups such as amino (–NH_2), carboxyl (–COOH), hydroxyl (–OH) or sulfhydril (–SH) is necessary (Nunes, 2005). Both CIP and ENR have a carboxyl group on carbon 7, enabling them to be conjugated to BSA and mcKLH. Initially the EDC reacts with the carboxyl molecule forming an intermediary reactive amine O–acylisourea. This intermediary amine can react with the amine group from the mcKLH although this reaction is unstable, being susceptible to hydrolysis in aqueous solution. When NHS (5mM) is added, it stabilizes the reaction converting the intermediary amide into NHS ester thus increasing the EDC efficiency.

According to the regular kit procedure the Imject® mcKLH and the Imject® BSA were reconstituted in 200 µL of MilliQ H_2O. One solution with either 2 mg of each antibiotic diluted in 500 µL of Imject® EDC Conjugation Buffer was mixed with either 100 µL of mcKLH or BSA. Then 10 mg of EDC was reconstituted in 1 mL of distilled H_2O and 50 µL of EDC solution was added to the antibiotic solution containing either mcKLH or BSA. The vials were incubated for 2 h at room temperature under constant shacking.

The material was then centrifuged and the supernatant purified in D–Salt™ Dextran Desalting Columns. Here the column was equilibrated with 20 mL of Imject® Purification Buffer Salts and the supernatant passed through the column; 21 samples containing 500 µL of the eluent were collected in eppendorf vials and read in spectrophotometer (Perkin Elmer, lambda EZ 150 UV/VIS) at 280 nm to evaluate the efficiency of the conjugation.

2.1.2. Recommendations

After collecting the fractions it is necessary to evaluate the eluate from the conjugation on a UV–Vis (using for example a Cary 50 Probe Varian/Agilent) the spectra of isolated compounds (antigen, carrier protein and the eluate). The ENR–KLH combination had a higher spectrum (~1.5 Abs, 265 nm) than ENR without KLH (~0.8 Abs, 265 nm). This helps to

ensure that the antigen is really bound to the carrier protein before it is inoculated into the target animal.

2.1.3. Rabbit inoculation

Before the first immunization, blood was collected from the marginal ear artery as negative control. Male castrated rabbits from pet stores were included (excluding New Zealand white). Where necessary, male rabbits were castrated at 4 weeks of age. Animals vaccinated against myxomatosis before 4 weeks of age were considered. Rabbits kept on substrate bedding/litter rather than in cages), became less aggressive and produced better antibodies.

The immunization was made through intradermal injection on the back side using 300 pg of antigen linked to BSA with adjuvant. The adjuvant was mixed with the antigen+BSA before inoculation. Under constant agitation on a vortex mixer, the adjuvant was added slowly (drip wise) into the vial containing the antigen. After mixing a syringe with a needle was used several times to emulsify the mixture. The intradermal injection was made with an insulin syringe and 27 G needle ¾ "(0.4×19 mm) BD 302200. The needle was securely attached to the syringe so the adjuvant to avoid spillage/splashing.

For the production of anti–rabbit IgG, goat was preferred to sheep. The procedure for goat inoculation is the same for the rabbits. Immunization could be performed every 4 weeks for four times and thereafter to 5 or 6 weeks. After the 4^{th} immunization, blood was collected with EDTA–aspirin mixer. Plasma was preferred to serum. The blood was collected between one week and three weeks after each immunization, at the peak of antibody production.

Rabbits were bled from the ear artery (to keep the rabbit alive long enough, and to enable inoculation of the booster at least every 2 m). It was possible to verify the increase in dilution titre with inoculation. The optical densities for ENR where on average close to 1 in the 4 inoculations for all 3 animals unlike CIP where results of the 3^{rd} and 4^{th} days inoculations were the closest (OD of 0.5–0.7). For CIP, in the 3^{rd} animal inoculated, the levels were close to 0.25. A main advantage of this procedure as increased dilution, besides using less rabbit serum, is precluding antibody purification or precipitation.

2.1.4. Rabbit serum IgG titration and indirect ELISA

The immunoassays to evaluate the reaction were done in polystyrene flat bottom plates (Nunc–Immuno Plate Maxisorp™ surface™) previously coated with 100 µL of ENR–KLH diluted at 1 µg/well in carbonate/bicarbonate buffer 0.1M at pH 9.6 incubated for two hours at 37°C and then overnight at 4°C. After incubation the liquid was removed, the plates washed three times with PBS–Tw (PBS, 0.05% Tween 20 pH 7.2) and blocked with 200 µL of casein 2.5% and incubated for 1 h at 37°C. The liquid was removed and the plates were washed again.

The sera collected from immunized rabbits were then diluted in PBS (between 1/200 and 1/3,200) and 100 µL added to the plates incubated for 2 h at 37°C. After incubation, the plates were washed with PBS–Tw 4 times, dried by ‘slapping’ on absorbent paper until the liquid was totally removed. Then 100 µL of anti–immunoglobulin conjugated rabbit IgG labeled with peroxidase diluted at 1/10.000 were added to the plates and incubated for 1 h at 37°C. After incubation, four more washes with PBS–Tw were performed, tetramethylbenzene (TMB) added as chromogenic substrate and the plate incubated for 10 min away from light. The reaction was stopped using sulphuric acid (H_2SO_4) 2M, prior to evaluation on an ELISA

plate reader (LabSystems Original MiltiSkan EX) at 450 nm wave length for colour evaluation.

The possible effects of different antigen (ENR, ENR+BSA and BSA) on binding of the antibody on the plate were evaluated. For instance, the effect of the antigens and the antibody separation from serum using ammonium sulphate precipitation showed exponential decrease in OD values from 2.4 to almost zero at the polyclonal antibody dilutions of 1:100, 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, 1:12,800, 1:25,600, 1:51,200, 1:102,400.

2.2.MONOCLONAL ANTIBODY EVALUATION

For high specificity and comparison purposes, monoclonal antibodies against ENR were obtained from RheaBiotech (Brazil). Local production of a monoclonal antibody proved very challenging after a year of trying due to low immunogenicity of the antigen. An enzyme immunoassay was used to evaluate the monoclonal antibody affinity and possible cross reactions, according to the described protocol. Polystyrene plates (Nunc–Immuno ™ Plate Maxisorp™ surface) were coated with 100 µL of antigens ENR–BSA, ENR–KLH, KLH and BSA diluted with 1 µg/well carbonate/bicarbonate buffer 0.1M pH 9 and incubated first for two hours at 37°C and then overnight at 4°C. After sensitization and drying, the plates were washed three times with PBS–Tw (PBS, 0.05% Tween 20, pH 7.2) and blocked with 250 µL of 2.5% casein and incubated for one hour at 37ºC. Excess liquid was then discarded and plates washed as previously described.

The anti–ENR monoclonal antibody was diluted (1/500, 1/1000, 1/2000, 1/2500, 1/4000 and 1/5000) in PBS and 100 µL added to the plate which was incubated for 2 h at 37°C. Excess liquid was discarded and the plates were washed with PBS–Tw 4 times. The plates were dried and 100 µL of 1/5000 peroxidase–labeled anti–mouse IgG (Fc specific–Sigma A2554) pipetted into the plates followed by incubation for 1 h at 37°C prior to washing 4 more times with PBS–Tw. As the chromogenic substrate, TMB was added and the mixture incubated for 10 min in the dark. The reaction was stopped with 2M H_2SO_4, and the colour variation read on an ELISA plate reader at 450 nm. Antibody KHL interaction was observed and showed cross reactivity of different monoclonal antibody dilutions produced against ENR challenged with different antigens bound to the plate.

2.3.EXPERIMENT TO DETECT RESIDUES IN BROILER CHICKEN

In order to have biological material to evaluate and validate the ELISA under development, an *in–vivo* experiment was conducted to obtain samples with different amounts of ENR with known concentrations. This *in–vivo* experiment aimed to also evaluate residues of CIP (the ENR metabolite). It was designed to evaluate feed consumption and live weight of broilers fed with different antimicrobial concentrations. To emphasize the antibiotic effect on the birds, they were kept on a used litter. The experiment was conducted in an acclimatized shed in Animal Science Experimental Sector, UNESP–Campus Araçatuba (Table 1).

TABLE 1. *IN–VIVO* EXPERIMENTAL DESIGN WITH BROILERS (DF–DEGREE OF FREEDOM)

	D.F.	D.F.	D.F.
Treatment	3	3	3
Error	12	12	15
Total	14	14	17

One day old broilers (n=336, Cobb) vaccinated against Marek's disease, Gumboro and Bouba flu, were used. The birds were weighed and housed in cages (boxes) properly adapted to the experimental conditions. Water and feed was supplied *ad libitum* with pressure cups that were later replaced by pendular drinker and feeder tube. The feed was balanced and free of any antimicrobial before treatment start [11].

Enrofloxacin at 3 mg/mL, 7 mg/mL and 10 mg/mL as 10% solution Baytril® (Bayer) was added to feed, 6 replicates per treatment. Three control groups were included. The diets containing antimicrobial were used for 10 consecutive days. From 20 days of age, exhaustion fans and evaporative plates were used for thermal comfort of the birds, a lighting program of 23 h light and 1 h dark was used with fluorescent lamps of 32 watt.

During the initial 24 days all birds were supplemented with an antimicrobial-free control diet. Birds were weighed on day 1 of life and at 24 days and after which slaughter weights were recorded. At the second experimental stage (‘antimicrobial phase’) animals were supplemented for 10 consecutive days with food/feed containing different amounts of antibiotics.

During these 10 days, three slaughters were made and also blood, kidney, liver and muscle tissue samples collected. These samples were used as known positive and negative samples in the competitive ELISA and Dot–ELISA–Multi standardization. In each study period (7, 21 and 41 days of age), bird performance was evaluated using as a reference, the initial and final weight (g), weight gain (g/d), feed intake (g/d) and feed conversion (g/g). Daily mortality rate and individual deaths weight were recorded to correct the feed conversion rates. Analysis of variance was done using the generalized linear model with a Statistical Analysis System (SAS) [12].

3. RESULTS AND DISCUSSIONS

3.1.BROILERS EXPERIMENT

Growth performance and feed consumption were compared for birds fed with different amount of ENR: 3 mg/kg, 7 mg/kg and 10 mg/kg Baytril® Solution 10% (Bayer) and the control group (no treatments). Total bird feed consumption (kg) during the initial phase (1:23 days) of the study, growth phase (1:33 days) and finishing phase (1: 40 days) in groups fed with different amounts of ENR averaged 1,500 kg, 3,000 kg and 5,000 kg, respectively. The corresponding bird weights (g) during the three experimental phases, initial phase (1:23 days), growth phase (1:33 days) and finishing phase (1:40 days) were 1,500 mg, 3,000 mg and 5,000 mg, respectively. Food consumption and conversion was not affected by the different concentrations of Baytril 10% (Bayer) in the treated group compared to the control group. For

the initial phase (1: 23 days), growth phase (1:33 days) and finishing phase (1:40 days) the ~1.3 kg, 1.5 kg, 1.7 kg, respectively.

No differences in live weight or feed conversion were observed between antibiotic treated broilers and the control group. Intensively reared broilers given animal feed with dietary additives are known to be healthier and less susceptible to disease (than those not fed) [4]. However, benefits of the antimicrobial additives depend on other factors e.g. quality of housing and installation conditions, climate, health and management. Since this experiment was conducted in a controlled environment, it is not possible to extrapolate the effects of antimicrobial action to other birds in an uncontrolled environment.

3.2.LC–MS/MS WORK

Biological material collected from the *in–vivo* experiment were analysed using a validated LC–MS/MS technique at the Thompson Mass Spectrometry Laboratory at Campinas-SP to determine the level of ENR and CIP to support the ELISA method development. Plasma, muscle, kidney, and liver samples collected at different periods of exposure/dosing with the antimicrobial were analysed. The results were as expected based on the antibiotic variation, and withdrawal periods/clearance time. The plasma ENR, CIP and ENR+CIP concentrations (ng/g) were almost zero by days 2–10.

Broilers were slaughtered at different times, during the treatment (10 days and 5 days) and immediately after the end of the treatment (day zero) and afterwards daily until 10 days after day zero. The LC–MS/MS test was performed on a pool of 6 samples per treatment group each day. As expected from the experimental design, the antimicrobial concentrations were elevated during treatment and higher in groups that received greater doses. These decreased as soon as the treatment was stopped. Plasma was useful in evaluating transfer of the drugs.

The drug concentrations in liver samples from broilers exposed to different concentrations, and slaughtered on different days during the treatment period, and daily thereafter were higher than in muscles. As expected, both drug levels were higher at treatment and decreased thereafter as soon as the treatment ended and demonstrating that drugs appear to accumulate in muscle compared to plasma. In plasma the ENR concentration increased from 950 ng/g to 1,000 ng/g and then 1,200 ng/g as drug levels in diet were increased from 7 mg/kg to 10 mg/kg and 13 mg/kg. The concentration was much higher in muscle (from 2,500 ng/g to 3,500 ng/g and 3,750 ng/g).

By the third day after drug withdrawal, all groups showed concentrations below the MRL. This indicates that 3 days is enough to ensure that residues of ENR are below permitted levels when the dosages of 7 mg/kg, 10 mg/kg and 13 mg/kg are administered for 10 days in the diet. A depletion curve of ENR in muscle, kidney, liver and plasma was developed. Validation data for the LC–MS/MS method were also produced (Annex II).

4. CONCLUSION

The successful production of antibodies involves the proper preparation of the immunogen with functional groups capable of being conjugated with a carrier protein when unable to generate antibody production especially as small molecules.

The study indicates that enzyme immunoassays have become increasingly refined and simple to perform. However, to ensure quality results, strict control over the standardization of antigen concentrations, antibody specificity, conjugates, time, and temperature among others

is required. There is a great need for faster, efficient and cost effective methods that can be used to detect very small amounts of residues in products such as milk, meat, eggs and honey.

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ANNEX I.

ANTIGEN PREPARATION FOR IMMUNIZATION

I.1. ANTIGEN PREPARATION

The immune system is able to respond to an antigen with the action and interaction of antigen–presenting cells, B lymphocytes and T lymphocytes. When these "strange" molecules come into contact with the immune system, it recognizes them and reacts against them. In this situation, the antigen is called an immunogen, resulting on the production of antibodies by B lymphocytes, associated to activation of T cells and generation of memory cells which function is to eliminate or limit the spread of the antigen.

Most strange protein and peptide with high molecular weight are natural immunogens, or are capable of triggering an immune response without any change in its chemical structure.

As ENR and CIP have low molecular weight (359.40 kDa and 331.4 kDa, respectively) the need of coupling to a carrier protein molecules with high molecular weight is required.

Many small antigens when conjugated to carrier proteins gain immunogenicity, and when inoculated into animals, stimulate the production of antibodies against both the antigen and the carrier protein. There are many options of proteins that can be used as carriers such as keyhole limpet hemocyanin (KLH 4.5×105 to 1.3×107 Da), bovine serum albumin (BSA–6,7000 Da) and ovalbumin (OVA–45000 Da). Basically they differ in immunogenicity, solubility and availability of functional groups that will conjugate with the small antigen.

The Mariculture KLH (mcKLH) carrier protein is enhances the production of antibodies, primarily by being derivative of a mollusc, it is phylogenetically different from other mammalian species and thus highly immunogenic. The mcKLH have hundreds of primary amines and carboxylic groups which can be conjugated via glutaraldehyde, EDC and NHS–ester as well as other reactants.

Thus we used the reagent Imject® Immunogen EDC Kit with BSA and mcKLH company PIERCE BIOTECHNOLOGY (Rockford, USA) to covalently conjugate enrofloxacin and ciprofloxacin to mcKLH and BSA. The peak absorbance for the ENR–KLH is distinct from the peak of ENR and KLH alone, suggesting a successful KLH conjugation. The coating of the carrier protein BSA with antigen gave the UV pattern similar to the BSA immunogen.

I.2. IMMUNIZATION PROTOCOL

I.2.1. Antigen preparation

First, the antigen (ENR or CIP) was diluted with the equal volume of complete Freud's adjuvant while stirring for 30 min; the same process was performed when using Freud's incomplete adjuvant.

I.2.2. Immunization cycles in rabbits

Before the start of each immunization cycle, blood from the marginal ear artery of each rabbit was collected. Then, 4 animals were immunized (intradermic injection at different locations) with mcKLH–ENR emulsified in Freud's complete adjuvant. Subsequent immunizations were

made at 15 days intervals and thereafter Freud's incomplete adjuvant was used with immunizations in the lumbar region.

I.2.3. ELISA standardization for IgG detection from rabbit serum

The kinetics of antibodies identified in rabbit sera collected at 0, 1 (15 days), 2 (30 days), 3 (45 days), 4 (60 days) and 5 (75 days) from the immunized animals was evaluated by immunoassays, using the following protocol. Briefly, polystyrene plates (Nunc–Immuno ™ Maxisorp ™ Surface Plate) were coated with 100 µL of antigen ENR–BSA diluted at 2 µg/ml in carbonate/bicarbonate buffer 0.1M pH 9 incubated overnight at 4°C. After sensitization and disposal of excess liquid the plates were washed three times with PBS–Tw (PBS buffer, 0.05% Tween 20, pH 7.2) and blocked with 200 µL 2.5% casein, incubated for hours at 37°C. After this period all excess liquid was discarded and plates washed.

Then, 100 µL of sera collected from the immunized animals were diluted 1/1,000 and 1/2,000 in PBS buffer added to the plates which were incubated for two hours at a constant temperature of 37°C. The excess fluid was discarded and the plates were washed with PBS–Tw 4 times. The plates were dried by beating up on absorbent paper until the excess liquid was removed. Next, 100 µLof conjugated anti–rabbit IgG immunoglobulin peroxidase labeled 1/10 000 were pipetted into the plates and incubated for 1 h at constant temperature of 37°C. After the end of this incubation, PBS–Tw was used to wash the plates, and the reaction shown using a chromogenic substrate as TMB following incubation for 10 min away from light. The reaction was quenched using a solution of 2M, sulphuric acid (H_2SO_4) and the reading done at 450 nm on the ELISA reader (SUNRISE, TECAN).

I.3. SEARCH RESULTS FOR PRODUCTION OF IgG BY ELISA IN RABBIT SERUM

Assays for standardization of ELISA were performed in parallel immunizations.

The main objective of the development of an immunoassay was to achieve maximum recognition by the antibody against a desired antigen. To be able to achieve this goal, six immunizations were performed on each animal to trigger reaction a response against ENR and CIP, with biweekly blood samples collected for partial assessments, which were subsequently titrated in indirect immunoassays. The titres were obtained from rabbit sera harvested as 0, t1 (15 days), t2 (30 days), t3 (45 days) and t4 (60 days), t5 (75 days). The ELISA response (OD) when using the sera for ENR ranged from 0.3 (1/100 dilution) to 1.2 (1/1,000). The level droped to ~1 at the highest dilution (1/2,000).

The dilution T0 (1/100) showed no reactivity with the antigen and levels increased over the weeks of immunization, demonstrating a good stimulation of the immune response against these antigens. However, when analysed individually, animal 3 was the most stable with regard to antibody production. Thus polyclonal anti ENR produced by the animal 3 was used**.**

I.4. IMMUNOAFFINITY PURIFICATION BY POLYCLONAL ANTI–KLH–ENROFLOXACIN

Fast Protein Liquid Chromatography (FPLC) consists of an LC system often used to quickly and reliably analyse, identify and purify proteins and peptides. This purification is possible due to the fact that each component of the mixture has preferably different stationary phase interactions (solid porous gel resin) or the mobile phase.

Purification was performed by affinity chromatography using Äkta Purification System GE Healthcare, Hi Trap Protein G HP chromatographic column (20mM sodium phosphate, pH 7.0). The column was initially equilibrated with binding buffer, following the injection of the serum of animal 3, enabling the specific (but reversible) binding of the protein to the column matrix. Five volumes of wash buffer and the target protein were recovered from the column by the gradual addition of the competitive ligand present in the elution buffer (0.1M glycine–HCl, pH 2.7). Thus the protein was collected in a purified and concentrated form. After elution, the column was re–equilibrated with affinity binding buffer and stored in 20% ethanol.

The visualization of the purified product was done in SDS–PAGE gel with 12% 1X Tris–Glycine buffer (25mM Tris base, 250mM glycine and 0.1% SDS). The electrophoresis conditions were: 180 V, 250 mA for 60 min. A mixture of 20 µL of sample with 5 µL of 5 loading buffer (5 × sample buffer, Thermo Scientific) was applied. The gel was stained with Page Blue^{TM} Protein Staining Solution (Thermo Scientific) following the Fast Protocol Staining protocol for mini–gels. Quantification of proteins obtained from the previous procedure was done and subsequent dialysis was performed in phosphate buffer for 48 h to remove excess salt in the sample. A protein peak was noted with affinity in the range of 2,800 mAU.

I.5. DETERMINING DILUTION OF POLYCLONAL ANTIBODY AND ANTIGEN FOR OPTIMAL COMPETITION ELISA

This assay was performed by ELISA in which polystyrene background (Nunc–Immuno ™ Plate Maxisorp™ surface) plates were coated with 100 µL of antigen–ENR BSA concentrations: 10 µg/mL; 5 µg/mL; 2.5 µg/mL; 1.25 µg/mL; 0.625 µg/mL and 0.3125 µg/mL diluted in carbonate/bicarbonate buffer 0.1M pH 9, and incubated overnight at 4°C. After sensitization and disposal of surplus liquid, plates were washed three times with PBS–Tw (PBS, 0.05% Tween 20 pH 7.2) and blocked with 200 of 2.5% casein, incubated for an hour at 37°C. All excess liquid was discarded and plates washed as before.

Then, 100 µL of polyclonal anti–enrofloxacin serially diluted (1/62.5 to 1/102,600) in PBS buffer was added to the plates and incubated for 2 h at a constant temperature of 37°C. The excess liquid was discarded and the plates were washed with PBS-Tw 4 times. The plates were dried beating up on absorbent paper until the excess liquid was eliminated. Next, 100 µL of conjugated anti–rabbit IgG immunoglobulin peroxidase labeled at 1/10,000 were pipetted into the plates and incubated for 1 h at constant temperature of 37°C. After incubation, four more washes with PBS–Tw were carried out the reaction revealed using as chromogenic substrate TMB incubated for 10 min away from light. The reaction was stopped using 2M H_2SO_4, before reading at 450 nm on an ELISA plate reader.

There was linearity with respect to the data analysed, showing a strong correlation between the various dilutions of antigen and antibody (Table I-1).

TABLE I-1. DETERMINATION OF OPTIMAL DILUTION OF ANTIGEN SENSITIZATION AND DILUTION OF POLYCLONAL ANTI-ENRO

Antibody polyclonal ENR-BSA	Antibody polyclonal
0.3125 µg/mL	1/1000
1.25µg/mL	1/2000
5 µg/mL	1/4000

I.6. CONJUGATION OF POLYCLONAL ANTIBODY TO COLLOIDAL GOLD TO BE USED IN IMMUNOCHROMATOGRAPHIC ASSAY

In the immunochromatographic test technique, also known as lateral flow assay is a detection method based on the use of strips of a support the impregnated material with dried reagents that are activated with the addition of fluid samples. Use for this methodology includes testing for pathogens, drugs, hormones and metabolites present in medical, veterinary, food, environmental analysis and other samples.

Basically, the test consists of two antibodies with distinct binding epitopes present in a sample. One of antibodies, the detection antibody, is labeled with a signal generator, in this case colloidal gold, and the other, which is called the capture antibody are immobilized on a solid surface. The detection antibody is inserted in a dehydrated state in a glass fibre membrane that will be dissolved upon contact with an aqueous medium containing the analyte of interest. By capillarity, this complex will migrate to be captured by the antibody previously adhered to nitrocellulose membrane. A cellulose fibre must be attached at both ends of the system to induce an immune complex absorption. The signal can be seen between 15 and 20 min after the process is started and can either be quantitative or qualitative to the molecule of interest to be assessed.

I.6.1. Results from a combination of immunoassay

The colloidal gold labeled anti–ENR polyclonal was prepared according to Olivier [1]. Briefly a colloidal gold solution was adjusted to pH 9.0 and 0.2 mol/L with sodium carbonate. The optimum protein concentration for labelling was determined viz: 10 µL of anti–ENR poly solution was serially diluted in borate buffer 2mM, and then 100 µL of colloidal gold solution added. Mixtures were incubated for 15 min at room temperature, and then 11 µL of 10% NaCl solution was added. The colour of samples changes from brilliant red to blue as the concentration of polyclonal antibody decreases. The optimum concentration of polyclonal for colloidal gold labelling was the lowest concentration of poly solution that did not change colour.

Later 150 mL of poly was incubated with 10 mL of colloidal gold solution (pH 9.0) for 30 min at room temperature. After adding 1 mL of 10% BSA solution in 20 mmol/L sodium borate (pH 9.0), the mixture was incubated at room temperature for another 30 min, and the labeled polyclonal antibody washed by repeated centrifugation (15,000g) at 4°C for 20 min. The supernatant was discharged and the pellet resuspended in 2 mL of 2% BSA and centrifuged (12,000g) at 4°C for 20 min. The second precipitate was resuspended in the buffer containing 3% BSA, 3% Sucrose, 0.01M sodium borate and 0.05% sodium azide and stored at 4°C for use.

I.6.2. Preparation of the conjugate pad

The conjugate solution was prepared by dilution of the colloidal gold labeled antibody to ENR. A conjugate pad was made by dipping a 1×0.5 cm glass fibre (Millipore) in the conjugate solution and then drying in a desiccator at room temperature.

I.6.3. Immobilization of capture reagents

The ENR–BSA (1 mg/mL) and goat anti–rabbit IgG (1 mg/mL) were applied to the nitrocellulose membrane using a dispenser as the test and control lines, respectively. The test

and control lines were situated 0.5 cm apart at the centre of the membrane and then dried, sealed, and stored under dry conditions.

I.6.4. Preparation of sample pad and absorbent pad

Sample and absorbent pads of Millipore were made from nonwoven, 100% pure cellulose fibre. The sample pad was cut to 2×0.5 cm and saturated with a buffer (pH 7.5) containing Tris–HCL and washed once with Na_2HPO_4 10mM (pH 7.5) and then dried at 37°C for use and stored as described above. The absorbent pad was cut to 2×0.5 cm.

I.6.5. Test procedure and principle

For the test 1.5 μL of standard solution or sample extract was added onto the sample pad, and the solution migrated toward the absorbent pad; the result could be seen after 10 min. When ENR was absent from the sample, all of the detection reagent would be trapped by capture reagent to form an easily visible test line. When ENR is present in the sample, it competes with the immobilized capture reagent for the limited amount of detection reagent. The more ENR is present in the sample, the weaker the test line colour. If sufficient ENR is present in the sample, it will completely block the reaction with the capture reagent. Thus, there is no visible test line on the nitrocellulose membrane. Provided that the test strip and the test procedure are correct, the control line is always visible. If no coloured capture line or only a red line at the test line appears, the testing procedure was likely improper or the strip invalid, and the test should be repeated using a new strip.

ANNEX II.
MS VALIDATION DATA

II-1. INTRODUCTION

One of the most prominent human health risks associated with intensive animal farming and antibiotic use is antimicrobial resistance. In particular, use of fluoroquinolones (FQs) in animals has generated growing concern because microbial resistance to these drugs has increased. The World Health Organization (WHO) and Food and Drug Administration (FDA) have placed severe restrictions on veterinary use of FQs, given the concerns about drug–resistant bacteria and the possible failure of human antibiotic therapy [1–3]. The fluoroquinolone ENR developed exclusively for veterinary use is advocated for treatment of chronic respiratory diseases, colibacillosis, salmonellosis and fowl cholera in large-scale poultry, where as other FQs, for example norfloxacin (NOR) and CIP, are used in human medicine only. However, CIP is the major metabolite of ENR (the de–ethylated product) and occurs in foodstuffs [4]. To safeguard human health, the EU has established safe maximum residue levels (MRLs) for veterinary medicinal products in foodstuffs of animal origin at the Community level under Council Regulation 2377/90 EEC and its later modifications [5]. Therefore powerful analytical methods for surveillance of possible residues are mandatory.

Various methods have been established for determining ENR and CIP in food-producing animals. Typically, these methods employ high–performance HPLC–UV and FLD, [6–8] detection or mass spectrometric [9–12]. This is often due to the co–elution properties for compounds belonging to the same chemical family and also due to pKa differences between the acidic and the amphoteric ENR and CIP [13]. Strict guidelines in European Union (EU) for confirmatory techniques state that LC–MS/MS monitoring two transition–product ions gives sufficient data to confirm the identity of a residue [5]. A tandem MS is capable of multiple–analyte detections.

The study aimed at developing a multi–residue method for screening and confirming ENR and CIP in various chicken samples by triple–quadrupole mass spectrometry after solid phase extraction. Method validation was performed for each matrix and the parameters including selectivity, accuracy and matrix effect studied.

II-2. MATERIAL AND METHODS

II-2.1. Blank and chicken samples

Chicken blank samples (plasma, muscle, kidney and liver) from Agrias group (Agrias commerce, Campinas, SP, Brazil), analysed to confirm absence of antibiotics and used to prepare matrix–matched calibration standards and fortified samples [14].

The depletion study was performed using 336 broilers (randomly divided into three groups) at one day of age and vaccinated against Marek's disease, Gumboro and Bouba flu. These birds were weighed and housed in cages (boxes) properly adapted to the experimental conditions. Water and feed were supplied *ad libitum* with pressure cups that were ultimately replaced by pendular drinker and feeder tube. The feed was balanced and free of any antimicrobial before treatment started [14].

Three different amounts of ENR 3 mg/mL, 7 mg/mL and 10 mg/mL of 10% solution Baytril® (Bayer) were provided with feed [14]. This involved 6 replicates per treatment and three control groups. The diets containing the antimicrobial were used for 10 consecutive days.

From 20 days of age, exhaustion fans and evaporative plates were then used for the thermal comfort of the birds. Fluorescent lamps of 32 watt were used in a lighting program of 23 h light (with 1 h of darkness). During the initial 24 days, all birds were supplemented with the control diet free of any antimicrobial. They were then weighed on day 1 of life and at 24 days after which slaughter weights were recorded. In the second experimental stage (phase antimicrobial) the birds were supplemented with different amounts of antimicrobial in feed for 10 consecutive days.

During these 10 days, three slaughters were done and blood sample, kidney, liver, muscle samples tissues collected. These samples were used as known positive and negative samples for the competitive ELISA and Dot–ELISA–Multi standardization. In each study period (7, 21 and 41 days of age), bird performance was evaluated using as a reference, initial and final weight (g), weight gain (g/d), feed intake (g/d) and feed conversion (g/g). Daily mortality rates and individual death weights were recorded to correct for feed conversion rates. Performance data were then analysed for variance using the GraphPad Prism 5, 2012 [14].

II-2.2. Chemicals and reagents

Standards of ENR, CIP were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) and CIP–d8 hydrochloride hydrate (internal standard, IS) was used. HPLC–grade methanol (MeOH), acetonitrile (MeCN) and hexane were purchased from Mallinckrodt (Hazelwood, MO, USA) while formic acid 98%, ammonium hydroxide (NH_4OH) 25% and ethylenediaminetetraacetic acid (EDTA 5mM) purchased from Sigma–Aldrich (St. Louis, MO, USA) were also used. SPE cartridges Strata–X polymeric Reversed Phase (60 mg/3 mL), were purchased from Phenomenex (Torrance, CA, USA). Water was purified by distillation and passed through a Milli Q system (Millipore, Bedford, MA, USA).

II-2.3. Standard solutions

Stock standard solutions (5,000 µg/mL) were prepared by dissolving the analytes in MeCN:H_2O (v/v) with 0.1% formic acid. The working standard solution containing all analytes with variable concentrations, according to their LOQ and MRL, was prepared by dilution of stock solutions in MeCN. Working Internal Standard (IS) solution of (20 µg/L) was prepared by dilution of stock standard solution (CIP d8) with MeCN. Stock standard solutions were kept in brown glass (to prevent photodegradation) and stored at -20ºC where they were stable for three months. Working solutions ENR and CIP were prepared in ACN with formic acid at a concentration of 100 µg/mL. All solutions were stored in dark glassware at -20ºC before analysis; working solutions were used for up to 3 weeks [14].

Spiking and calibration standards at various concentrations were prepared by combining aliquots of working solutions and IS with the LC mobile phase. These solutions were stored in amber coloured glassware at -20°C for up to 2 days. Calibration standard mixtures had the following final concentrations: 1 ng/mL, 2 ng/mL, 5 ng/mL, 10 ng/mL, 20 ng/mL, 50.75 ng/mL and 150 ng/mL (ENR/CIP) for plasma; 2 ng/mL, 5 ng/mL, 10 ng/mL, 50 ng/mL, 100 ng/mL, 150 ng/mL, 200 ng/mL and 350 ng/mL (CIP) and 2 ng/mL, 5 ng/mL, 8 ng/mL, 10 ng/mL, 30 ng/mL, 50 ng/mL, 80 ng/mL (CIP) for muscle; 5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 300 ng/mL and 500 ng/mL (ENR/CIP) for kidney and liver. These solutions were prepared daily from the stock solutions by serial dilutions. The IS concentration in all calibration standard mixtures and final sample solutions was 80 ng/mL. Tuning solutions (500 ng/mL) were freshly prepared in MeCN containing 0.1% formic acid [14].

II-2.4. Instrumentation

For sample preparation, an Eppendorf Centrifuge 5418 (Rotor FA–45–18–11, Hamburg, Germany) and mixer IKA® Vibrax VXR Basic (IKA Works Inc., Wilmington, NC), Manifold (Agilent Technologies 1260 series, Waldbronn, Germany) were utilized. For quantitation, an HPLC system (Agilent Technologies 1260 series, Waldbronn, Germany) coupled to a Q–TRAP 5500 tandem mass spectrometer (AB Sciex, Concord, ON, CA) equipped with electrospray (ESI) source were used. Data acquisition and processing was performed using the Analyst 1.6.1 and Multiquant 1.3.1 software package [14].

II-2.5. LC and MS/MS condition

Chromatographic separation of analytes was performed on a Kinetex PFP 100Å reversed phase column (00B–4462–E0, 100 mm×4.6 mm i.d., 2.6 µm particle size, Phenomenex, Torrance, CA, USA) with a compatible pre–column (PFP, PN AJ0–8773, Phenomenex, Torrance, CA) at a flow rate of 850 µL/min for 8.5 min and injection volume 10 µL. The autosampler was kept at 10ºC, the column at 30ºC and MeOH:H_2O:NH_4OH (5:4:1, v/v/v) was used during 45 sec for needle wash between samples. The isocratic mobile phase was composed of acetonitrile with 0.1% formic acid/H_2O with 0.1% formic acid (60:40, v/v).

The ESI source was operated in the positive ion mode (ESI+). Nitrogen was used as curtain (10 pounds pers square inch, psi), nebulizer (40 psi), auxiliary (15 psi) and collision (high or 12 a.u.) gas. The ion transfer voltage was set to 4,500 V, and the probe temperature to 650°C. Sample analysis was carried out in the multiple–reaction monitoring (MRM) mode with a dwell time of 50 msec per channel. Compounds and source–dependent parameter optimizations were performed by infusion of standard solutions. The most sensitive fragment signal was selected as the quantification ion pair, while the second highest fragment signal was selected as confirmation ion pair (Table II–1). All other conditions were optimized through HPLC injections.

TABLE II–1. OPTIMIZED MS/MS CONDITIONS OF ENR, CIP AND CIP-D8 [14]

Analyte	[M+H]	Fragment	Declustering potential (V)	Collision energy (V)	Entrance potential (V)	Collision cell exit potential (V)
ENR	359	245	146	37	10	16
		203		53	10	6
CIP	331	314	91	33	10	6
		231		49	10	12
CIP-D8	339	296	251	30	10	8

II-2.6. Sample, control and curve preparation

On average 0.5 g muscle or plasma tissue and 0.3 g kidney and liver tissue was accurately weighed and placed in a 2 mL Eppendorf tube 20 µL of 20 µg/g IS solution added and the mixture left to stand for 15 min at 5°C prior to adding 1,000 µL of MeOH with 0.1% formic acid. Appropriate stock solutions were added to blank samples for quality control (QC) purposes, and IS solutions added to the unknown samples. These were shaken on a vortex mixer for 10 min at 1,000 rpm, and the mixture centrifuged at 15,000 rpm for 5 min. The organic phases were transferred into a 15 mL falcon tube and the residue washed twice with 800 µL of MeOH and 0.1% formic acid and 12 mL H_2O to the organic extract.

The samples were cleaned with SPE cartridges activated with 2 mL of MeCN and 2 mL of H_2O. The cartridges were then washed with 2 mL of water and 3 mL of hexane. The two drugs (ENR and CIP) were eluted with 5 mL of mobile phase containing 5mM EDTA and 1,000 µL of the resulting solution kept in brown glass before injection into the LC–MS/MS system. The QC samples were eluted with 15 mL of mobile phase with 5mM EDTA. To 150 µL of the resulting solution, 850 µL of mobile phase with 5mM EDTA were added, the mixture kept in brown glass and later injected into the LC–MS/MS system. The calibration curve is extracted with blank tissue sample spiked by adding the appropriate standard solutions.

II-3. RESULTS AND DISCUSSION

II-3.1. Method validation

The developed method was applied to determine ENR and CIP in muscle, kidney, liver tissue and plasma chicken samples. Tissue and plasma blank samples were fortified with ENR and CIP at four concentration levels. Three sample extracts were injected at each level. The recovery averages from spiked chicken samples were determined at each concentration level by comparing the peak area of each compound with the respective calibration curve. These curves were obtained from the peak area ratio (y–axis) of analyte to IS, against the concentrations of analyte (x–axis) using Multiquant Software Version 1.3.1 (AB Sciex). The correlation coefficient values ($R^2 > 0.999$) indicated appropriate correlations between the investigated compound concentrations and their peak area within the test ranges (Table II–2)

TABLE II–2. RETENTION TIMES AND LINEARITY FOR ENR AND CIP STANDARDS IN CHICKEN TISSUE.

Tissue	analyte	Retention Time	R^2	Equation	Curve Range
Plasma	ENR	4.30±0.02	0.99865	y=0.00328x + 0.06223	1-150 pg/mL
	CIP	3.50±0.02	0.99975	y=0.00236x + 0.0843	1-150 pg/mL
Muscle	ENR	4.30±0.02	0.99907	y=0.00267x + (-) 0.01333	2-350 pg/mL
	CIP	3.50±0.02	0.99965	y=0.00468x + 0.04345	2-100 pg/mL
Kidney	ENR	4.30±0.02	0.99988	y=0.00188x + (-) 0.03567	5-500 pg/mL
	CIP	3.50±0.02	0.99934	y=0.00289x + 0.03223	5-500 pg/mL
Liver	ENR	4.30±0.02	0.99911	y=0.00234x+ 0.04678	5-500 pg/mL
	CIP	3.50±0.02	0.99922	y=0.00376x + 0.0363	5-500 pg/mL

Good chromatographic separation (figure II–1) of the ENR and CIP was achieved by using the following mobile phase gradient MeCN:H_2O with 0.1% formic acid (60:40, v/v). The efficiency of the SPE column clean–up was evaluated in comparison with liquid–liquid extraction (LLE) in terms of interferences [14].

In this study we used the PFP column and peak tailing/fronting were reduced using by adding EDTA in the mobile phase used for eluted ENR and CIP. Thus, as recommended by Lee et al [15], we added 5mM EDTA into mobile phase throughout this study and did not observe any negative effects in the ESI ionization efficiency for the fluroquinolones.

II-3.2. LOD and LOQ

The LOD and LOQ signal–to–noise (S/N) values were determined using five injections of muscle, kidney, liver, fat/skin tissue and plasma chicken samples extracts spiked with 5 ng/ mL for ENR and CIP. The LOD were calculated based on signals with 3 times S/N values and LOQ were calculated based on 10 times S/N values. The respective LOD/LOQ values for

ENR and CIP were (0.15/1.0 ng/g) for plasma, (0.15/2.0 ng/g) for muscle and (0.25/5 ng/g) for kidney and liver.

Matrix effect was investigated using muscle, kidney, liver, fat/skin tissue and plasma samples. Plasma samples contained 6, 100 and 2,600 ng/mL (ENR/CIP); muscle 3 ng/g, 45 ng/g and 4,000 ng/g (ENR) and 3 ng/g, 45 ng/g and 200 ng/g (CIP); kidney 6 ng/g, 290 ng/g and 3,000 ng/g (ENR and CIP) and liver 6 pg/ng, 290 pg/ng and 4,000 pg/ng (ENR and CIP). Peak areas of the IS prepared in mobile phase were compared with ENR and CIP standards in muscle, kidney, liver, fat/skin tissue and plasma samples extracts spiked at the same nominal concentrations. The matrix effects for plasma were 17%, 12% and 7% for ENR and 16%, 15% and 6% for CIP; for muscle 30%, 22% and 10% for ENR and 28%, 20% and 8% for CIP; kidney 38%, 30% and 18% for ENR and 34%, 27% and 15% for CIP and for liver 36%, 30% and 16% for ENR and 35%, 30% and 9% for CIP [14].

Quantification of analytes using matrix calibration curves is strongly recommended especially when ion signal suppression exists. Fortunately, the matrix effect in our study was less than in other FQs [16]. When using ESI ionization, the presence of matrix components (salts, proteins, lipids, carbohydrates, etc) that affect ionization of the target analytes may pose a significant problem, by either reducing or enhancing analytes response [17]. This may however be minimized by an efficient clean–up step (such as SPE) or appropriate chromatographic conditions.

II-3.3. Accuracy and precision

This was determined by analysis of low, medium, high and hyper–high dilution QC samples at four different concentrations injected first on the same day and assessed for intra–day variation, followed by inter–day assay following injection of three samples of each concentration on three days.

The percent relative standard deviation (% RSD) of the measured concentrations was used to report precision. The recoveries for all compounds were investigated at three concentration levels and they were between 93% and 111% for plasma, 99% and 116% for muscle, 99% and 119% for kidney and 99% and 117% for liver. Table II–3 reports intra–day and inter–day variation data (RSD) of chicken samples.

II-3.4. Stability

No significant degradation (less than 0.5%) of the standards was observed even after 24 h storage of muscle, kidney, liver, fat/skin and plasma samples at 10ºC on an autosampler tray. Nonetheless, at room temperature the degradation was higher than 10% after 24 h. The standard solution (150 ng/mL) diluted in MeCN with 0.1% formic acid degraded by 5% in four weeks at -20ºC. The calibration mixtures diluted in the mobile phase degraded by 5% (CIP) and 10% (ENR) in 72 h at 10ºC.

TABLE II–3. LC–MS/MS METHOD PERFORMANCE FOR ENR AND CIP.

Plasma ENR (ng/mL)	Intra (*n* = 3) mean + s (ng/mL)	CV (%)	Recovery (%)	Inter (*n* = 6) mean + s (ng/mL)	CV (%)	Recovery (%)
6	6 ± 0.53	8.83	100	6.67 ± 0.73	10.94	111
100	99 ± 6.08	6.14	99	105 ± 5.76	5.49	105
290	270 ± 8.18	3.03	93	288 ± 4.76	1.65	99
2600	2588 ± 76.34	2.95	99	2590 ± 93.7	3.66	99
Plasma CIP (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
6	6.3 ± 0.48	7.62	105	6.8 ± 0.68	10	113
100	105.6 ± 8.63	8.17	106	107 ± 10.4	9.7	107
290	298.6 ± 6.63	2.22	103	297 ± 8.4	2.83	102
2600	2659.4 ± 35.34	1.32	98	2677 ± 63.7	2.38	103
Liver ENR (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
6	6.1 ± 0.70	11.4	102	7 ± 0.79	11.28	117
180	188.8 ± 2.65	1.40	105	193 ± 5.8	3.01	107
290	295.2 ± 4.65	1.58	102	298.2 ± 1.75	0.59	103
4000	4008.9 ± 10.99	0.27	100	4033 ± 16.1	0.40	101
Liver CIP (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
6	6.3 ± 0.34	5.40	105	7 ± 0.78	11.14	116
180	182.5 ± 7.97	4.36	99	185 ± 11.4	6.16	103
290	298.3 ± 6.34	2.13	103	300.3 ± 8.34	2.78	104
4000	4058.2 ± 21.20	0.52	101	4088 ± 34.5	0.84	102
kidney ENR (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
6	7.1 ± 0.53	7.46	118	7.15 ± 0.88	12.30	119
120	124.5 ± 6.08	4.88	104	127.8 ± 7.76	6.07	107
290	295.3± 8.08	2.74	102	298.3± 9.08	3.04	103
6000	6020.5 ± 16.34	0.27	100	6050 ± 22.7	0.38	101
Kidney CIP (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
6	6.8 ± 0.48	7.06	113	6.9 ± 0.66	9.56	115
120	119.6 ± 10.63	8.88	99	122.8 ± 13.4	10.91	103
290	288.6 ± 8.63	2.99	99	290.6 ± 10.6	3.65	100
3000	3055.4 ± 25.34	0.83	102	3078 ± 36.7	1.19	103
Muscle ENR (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
3	3.1 ± 0.10	3.22	103	3.5 ± 0.23	6.57	116
45	44.8 ± 2.65	5.92	99	46.8 ± 3.8	8.11	104
90	92.6 ± 3.63	3.92	102	95.6 ± 5.63	5.88	106
4000	4080.9 ± 21.99	0.54	102	4099.3 ± 26.1	0.64	102
Muscle CIP (ng/mL)	**Intra (*n* = 3) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**	**Inter (*n* = 6) mean + s (ng/mL)**	**CV (%)**	**Recovery (%)**
3	3.1 ± 0.34	10.96	103	3.2 ± 0.45	14.06	106
45	43.5 ± 1.97	4.52	97	48.4 ± 4.4	9.09	108
90	94.6 ± 3.63	3.83	105	96.6 ± 4.63	4.79	107
200	205.2 ± 11.20	5.46	103	208.3 ± 14.5	6.96	104

II-3.5. ENR depletion study

Since broiler production may require prophylactic and/or therapeutic use of antimicrobials, it is necessary to have knowledge of the drug depletion in the animal tissue (to ensure that

residues are below maximum permitted limit). The average concentrations of ENR in the different groups, days after administration of the drug, as well as the relative standard deviations and confidence intervals ($P < 0.05$) in tissues showed an exponential decrease within the first 3 days and plateaus off until day 10 for all concentrations 7 mg/kg, 10 mg/kg and 13 mg/kg.

On the third day after drug withdrawal, all groups showed concentrations below the MRL. This indicates that a grace period prescribed above (3 days) for dosages of 7 mg/kg, 10 mg/kg and 13 mg/kg administered for 10 days in the diet is enough to ensure that residues of ENR are below permitted level at the time of consumption.

II-4. CONCLUSION

An accurate, reproducible and highly sensitive ESI LC–MS/MS method for testing ENR and CIP in five chicken matrices (plasma, muscle, fat/skin, kidney and liver) has been validated according to FDA and European Community guidelines. The detection and quantification limits were found to be low enough to determine ENR and CIP residues in chicken tissues below the permissible MRLs established by the European Medicines Agency. Broiler chickens were successfully used to determine depletion of ENR in muscle, kidney, liver and plasma. Thus for birds fed Baytril for 10 consecutive days at different concentrations (7 mg/kg, 10 mg/kg and 13 mg/kg) a grace period of 3 days was established when tissues for human consumption will contain tolerable levels of the drug residues.

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INVESTIGATING SUSPECTED NATURAL SOURCES OF CAP AND RESIDUES OF THE DRUG IN ANIMAL, PLANT AND SOIL SAMPLES IN MONGOLIA

ENKHTUYA, T

State Central Veterinary Laboratory of Mongolia, Ministry of Agriculture and Light Industry, Ulaanbaatar, Mongolia

Abstract

The presence of Chloramphenicol (CAP) in selected, plant, animal and environmental samples, has been investigated using the Enzyme Linked ImmunoSorbent Assay (ELISA) and High Performance Liquid Chromatography (HPLC) for screening and LC–MS/MS for confirmation. Varying levels of CAP were found in pastures such as *Artemisia* and *Thalictrum* species as well as meat and casings. Herbs growing on the Mongolian pastures do not always contain high concentrations of CAP and no single herb family is clearly associated with CAP that could end up in animal products. A large number of root samples contained the drug. However, it is not possible to associate specific plants as the exact sources.

1. INTRODUCTION

As a member country of the World Trade Organization, Mongolia has obligations to make national legislation consistent with provisions regarding food safety issues in international agreements such as Sanitary and Phytosanitary agreement, agreement on Technical Barriers to Trade and Codex Alimentarius.

Mongolia has a population of ~2.9 million and area of 1,564,116 km^2. Traditionally, nomadism is central to Mongolian culture, and most of the local population is in animal husbandry which is 20% of the GDP [1, 2]. This industry is not only one of the main sources of income but the main food source for Mongolians. It is thus important to ensure safety of animal products for public health and to safeguard trade. As of 2013, Mongolia's total livestock population was 45.1 million, including 2.9 million cattle, 23.2 million sheep, 19.2 million goats, 314,400 camels and 2.6 million horses [1, 2]. Swine and chicken are approximately 0.5 million though are less valued.

Currently international trade in Mongolian livestock sector is limited to an annual export of 10 000 ton of meat. However, there are opportunities to export animal derived products to countries including Russia, China, Southern East Asian and Arabian countries. Such opportunities require certification of animal health and safety requirements and veterinary drug residue monitoring, particularly of prohibited drugs such as CAP, thus presenting challenges.

Of all animal products, Mongolia exports only small intestine of sheep and goats processed as sausage casings to the EU. Exports of casings have had good international markets in the former Soviet Union and former Eastern European countries. Annually, 3.2 million pieces or approximately 80 million meter of small intestines are exported (for making sausage casings,) more than 80%–90% of which are destined for Western European countries, including Germany, the Netherlands, Italy and Switzerland.

In 2003, 2004 and 2006 CAP was detected (<0.3 mg/kg) in casings exported to EU affecting the market and necessitating CAP residue testing program for sheep casings was established [3, 4]. A research project on antibiotic residue testing in meat products was also initiated in 2009 through 2013/4. Thus this research aimed at investigating why CAP is detected in Mongolian meat, offal, and plants, and to generate knowledge and develop techniques for use by relevant organizations. This was the first comprehensive research on determination of CAP

residue levels/limits in Mongolian meat and by–products. A number of detection methods were developed, and contributed to establishment of a system for monitoring residues in animal products, as well as nation–wide standardization and use of testing methods.

The drug CAP is a broad–spectrum antibiotic from *Streptomyces venezuelae*, several other *actinomycetes* and also produced by chemical synthesis is commonly used in human and veterinary medicine [5]. Although potent, due to its several adverse effects, use in food animals is in tolerated by many countries [6–8]. The minimum required performance limit (MRPL) of 0.3 µg/kg was also assigned by the European Commission for analytical methods testing for CAP in products of animal origin [9] to ensure consumer protection. Residues of CAP have been reported in poultry, honey and sheep casings, impacting international trade although sources may not be clear and the drug may not have been used in production [10].

Analytical methods for CAP including gas chromatography and liquid chromatography combined with mass spectrometry (GC– or LC–MS) have been reported [11–15] and are capable of meeting the MRPL level of 0.3 µg/kg in various food products. Cheaper, rapid screening tools may be the more accessible tools for developing countries such as Mongolia. However, confirmatory tools including highly selective instruments are inevitable [16, 17]. Thus, a collaborative study was undertaken to analyse CAP in plant and environmental samples as well as animal products from Mongolia.

2. MATERIALS AND METHODS

Initial research work was conducted by the laboratory of Food hygiene and residue of the State Central Veterinary Laboratory of Mongolia (SCVL), and some confirmatory tests performed at RIKILT, Wageningen University, The Netherlands with some tests at a laboratory in Belfast.

2.1.PLANT AND ANIMAL TISSUE SAMPLES

Meat and intestinal samples were randomly collected from abattoirs in rural areas, meat and small intestine processing plants, some importing enterprises and food markets. The average sample weight was 500 g and each sample (as fresh as possible) was clearly recorded. Collected samples were transported in portable refrigerators at -4ºC to -8ºC. Cold chain systems for a local meat company (JUST AGRO Co Ltd) were used to collect meat samples in remote sources or the western region.

Bitter tasting plants were fed to sheep and the meat analysed for CAP. The plants including *Artemisia sieversiana*, *Artemisia*, *Artemisia frigida*, *Thalictrum simplex*, *Tanacetum vulgare L*, *Ledum palustre L*, *Rheum undulatum L*, *Thymus goblicus*, and *Thermopsis dagurica*, were collected in Zavkhan, Tuv, Dornogovi, Sukhbaatar, Bulgan and Selenge provinces. After collection, plants were sorted and dried in well ventilated, clean, dry and dark place. Dried raw material was then packaged in double paper bags, labelled and stored.

2.2.MAIN METHODS, KITS AND EQUIPMENT USED

For ELISA, various commercial ELISA kits and the Eurodiagnostics, EuroProxima 5091CAP method [18] used at SCVL as well as an inhouse method developed by RIKILT. Other tools included HPLC with a diode array detector (HPLC/DAD, Agilent) along with Novapac C18 (3.9×150 mm, 5 µm) and LC–QqQ–MS as well as UPLC–ToF–MS, (Waters, USA) for confirmation.

2.2.1. ELISA tests

The CAP–EIA kit [18] is a competitive enzyme immunoassay for screening and quantitative analysis and utilizes a specific antibody raised in rabbits against protein conjugated CAP. The cross–reaction levels are 100% to CAP, 65% to CAP–glucuronide, <1% to thiamphenicol, and <1% to florfenicol. The detection limit for tissue was 0.02 ng/g and 0.1 ng/g for feed; recovery rate ≥80%.

2.2.1.1.Sample treatment of meat and casings for ELISA test at SCVL

Ten grams of homogenized meat sample was weighed into 50 mL volume flask, 6 mL ethyl acetate was added, and the material was rigorously mixed on a shaker for 10 min. The material was then centrifuged at 2,000 rpm for 10 min, 4 mL of supernatant aspirated into a test tube and then dried by evaporation at 50°C. The dried residue was then reconstituted in 1 mL iso–octane/chloroform (2:3, v/v) and 1 mL sample diluting buffer solution was added, before mixing for 1 min. This material was then centrifuged at 2,000 rpm for 10 min and then 10 µL of the lower layer used for the ELISA test.

2.2.1.2.Preparation of herbs for ELISA test at SCVL

Here 10 to 100 g of herb sample was ground and 5 g homogenized in 20 mL of distilled water (H_2O). Five mL of the homogenate was pipetted into a glass tube and 10 mL of ethyl acetate added before mixing for 30 min followed by centrifugation for 10 min at 2,000g. Furthermore, 5 mL of the ethyl acetate (upper layer) was pipetted into a glass tube and evaporated at 50ºC under a mild stream of nitrogen. The fatty residue was dissolved in 0.5 mL of iso–octane/trichloromethane (2:3, v/v) and 0.5 mL of sample dilution buffer added. The combination was shaken using a vortex mixer for 1 min and centrifuged for 10 min at 2,000g. An aliquot of 50 µL of the upper layer was then used in the ELISA test.

2.2.2. Preparation of pasture plants for CAP analysis by ELISA at RIKILT

First, a plant was homogenized using a mixer and 1 g of grass transferred into tube followed by shaking in 20 mL H_2O and centrifuging for 15 min at 3,000g. The supernatant (4 mL) was transferred to a glass tube and the pH adjusted to 6.5–7.5 and 3 mL supernatant applied to an Extrelut®NT3 cartridge (Merk, Darmstadt, Germany). After 30 min of equilibration, elution was done with 15 mL dichloromethane into glass tube and evaporated to dryness with a stream of nitrogen. The residue in all tubes (standards and samples) was dissolved in 0.5 mL PBST and placed in an ultrasonic bath for 2 min and 250 µL later transferred to a dilution plate.

Then, coated plates are washed with washing–buffer and 50 µL (pre–diluted) sample added in duplicate into the EIA–plate (positions A4–H12) and 100 µL PBST (positions A1–A3, H1), 50 µL PBST (positions B1–G3). Standards (50 µL) were added (positions B1–G1) as well as 25 µL of HRP and the antibody. The plate was sealed, mixed for 2 min on a shaker and incubated overnight in a refrigerator. The plate was then washed with buffer and 100 µL of substrate solutions added and then stored in a dark at room temperature for 15 min after which 100 µL of 1M phosphoric acid was added and the contents mixed for 10 sec on a shaker. The plate was then read in the 96–well spectrophotometer (Reader type–Microplate Manager Bio–rad Laboratories, Model 550) at a wavelength of 450 nm.

2.2.3. Sample treatment for HPLC/DAD analysis

Five gram homogenized meat samples were transferred into 50 mL sample flask, 2 mL distilled water and 7 mL ethyl acetate added followed by rigorous shaking for 15 min. The mixture was centrifuged at 5,300 rpm for 15 min and the supernatant containing ethyl acetate separated. This step was repeated twice. The separated supernatant was then evaporated at 50°C to dryness, before suspension in 2 mL n–hexane/chloroform mixture at ratio 1:1 (v/v) followed by rigorous mixing. The mixture was centrifuged at 2,000 rpm for 10 min, supernatant separated and filtrated through 0.22 µm filter before HPLC analysis. The HPLC/DAD conditions included: Novapac C18 (3.9×150 mm, 5 µm); Wavelength: 278 nm; Mobile phase: 0.005M $(NH_4)_2HPO_4$/MeCN (80:20, v/v) and a flow speed of 1 mL/min.

2.3.FEEDING ANIMALS BITTER PLANTS

Animals were fed *A. sieversiana*, *A. frigida* and *Th. simplex L* for 7 to 14 days to evaluate if CAP would be detected in the animal tissues. Fourteen clinically healthy sheep of the same sex, ages and fattening levels were chosen for the experiment. Although the animals originated from Erdenekhairkhan district, Arkhangai province and Lun district of Tuv province, flocks were driven through the territories of Arkhangai, Bulgan and Tuv provinces and grazed in the area around Emeelt for 5 to 10 days.

After purchase, the sheep were kept in experimental sheds at the School of veterinary science and biotechnology (SVSB) in Mongolia. A sheep was fed *A. sieversiana* and another with *A. frigida* for 7 days. Three groups of sheep each consisting of 2 animals were fed with *A. sieversiana*, *A. frigida* and *Th. simplex,* respectively for 8–14 days in separate sheds. A sheep in each experimental group was treated as a control and fed hay free from the bitter plants, and 5 g capsulated therapeutic CAP (PHILCO, Germany) orally administered twice a day. Additionally, 3 healthy rabbits of the same sex, age and body weight and raised in Songino, were chosen for our experiment and aqueous extracts of both *A. sieversiana* and *A. frigida* administered orally at the Department of non–infectious diseases and pharmacology of SVSB. After the experiment, all sheep were slaughtered, muscle, liver and small intestinal samples collected and tested in the SCVL's laboratory for drug residue monitoring.

2.4.SURVEY OF THE WESTERN REGION MONGOLIA

Study samples were collected from 21 districts of 7 provinces (including Bayantes and Tsagaan chuluut, Zavkhan province, Burentogtokh, Ulaan–Uul and Jargalant of Khovsgol province, Khureemaral, Ulziit and Shinejinst, Bayankhongot province, Nogoonnuur, Tolbo and Sagsai, Bayan–Ulgii, and Malchin, Umnugobi and Undurkhangai, Uvs province). A total of 257 sheep, goats, cattle and horse samples were analysed for residues of CAP, antiparasitic drugs including avermectin/ivermectin, kainate, and radionuclides at SCVL’s laboratory of Food Hygiene and Residue Analysis. Instruments used included ELISA, HPLC and gamma spectrometry.

A semi–structured questionnaire was also administered among veterinarians, animal husbandry specialists, herders and civilians, working and living in the areas where bitter taste plants such as the large head wormwood (*A. macrocephala Jacquem*) were common. The questionnaire focused on pasture plants associated with a bitter taste in animal meat and milk; distribution; species and palatability of such plants, and whether CAP was used. Fifty veterinarians and animal husbandry specialists, over 100 herders and 30 consumers in Zavkhan, Bayankhongor, Sukhbaatar, Dornod, Tuv and Selenge provinces were interviewed.

For purposes of investigating CAP residues in small intestine from Mongolian livestock, a total of 135 people participated in the survey. These included raw material managers, workers and handlers in units and plants for collection of small intestines in Zavkhan, Tuv and Selenge provinces and Ulaanbaatar, and raw material markets in Nalaikh and Emeelt. The collection and handling process was also observed by the investigators in this study and workers interviewed. According to respondents, sheep and goats small intestines were mostly prepared for export, while those from larger livestock were mostly collected by sausage manufacturers from local meat plants. It was noted that although small intestines may be collected during any season most were prepared in autumn and early winter.

2.5.SURVEY OF THE CENTRAL REGION

A semi structured questionnaire was administered to determine commonly used veterinary drugs in the central region and respondents included veterinarians and animal health specialists working and living in rural areas. A total of 32 veterinarians and 27 other specialists in 27 districts of 9 provinces including Tuv, Selenge, Darkhan, Dornogobi, Orkhon, Gobisumer, Umnugobi and Uvurkhagai were included in the survey.

Meat and milk samples were also collected from districts in Darkhan–Uul, Selenge, Tuv, Govi–Sumber, Dornogovi, Dundgovi and Umnugovi aimgas, covering local animal husbandry and crop producing and major mining areas as well as major food markets in Ulaanbaatar and Darkhan cities. Up to 173 sheep, goat and cattle meat samples and 100 bovine milk samples were randomly collected. Furthermore, ELISA tests were used to analyse CAP in plants (20 species) from Selenge, Bulgan and Tuv provinces and some districts of Ulaanbaatar city, suspected of causing a specific taste/odour in animal products.

2.6.CONFIRMATION OF TEST SAMPLES IN THE NETHERLANDS

As reported elsewhere [10], plant samples were obtained from Mongolia, a local store in the Netherland, as well as a sample of *A. fridiga* from Utah, USA, and analysed for CAP by LC–MS/MS. Additional samples including leaves, roots, stalk of *A. sieversiana*, *A. frigida* as well as green grass, soil and water samples (n=192) were collected and sent from Mongolian to RIKILT. During the sampling in Lun, Atar, Hui doloon xudag, Erdene and Bayandelger districts, 3 different locations each were selected and from each location 3 samples were then split into leaves, roots and, if available, stalks [10].

2.6.1. Extraction and clean–up procedures of LC/HRLC–MS/MS analysis

Plants were homogenized using a mixer and 1 g of the material and 2 g of soil (5 replicates) weighed into 50 mL test tubes and to each sample, 20 mL H_2O and standards were added. The tubes were mixed for 30 min and centrifuged for 15 min at 3,000g, and 4 mL of the supernatant transferred into glass tube. Three mL supernatant was applied to an Extrelut® NT3 cartridge. After 30 min of equilibration, CAP was eluted with 15 mL dichloromethane into glass tube and evaporated to dryness with a stream of nitrogen at 35ºC. The residue in all tubes (standards and samples) was dissolved in 0.5 mL Milli–Q H_2O and placed in an ultrasonic bath for 2 min, centrifuged for 15 min at 3,000g and 2 mL toluene added and content vortexed. The bottom/aqueous layer was then transferred into an HPLC vial and analysed by LC–MS/MS [10].

The following were used: Ultra–performance liquid chromatography in combination with triple–quadrupole mass-spectrometric detection (UPLC–QqQ–MS); MS–grade solvents for extraction and clean–up Milli–Q system H_2O, MeCN (Merck), Dichloromethane (Merck),

Toluene (Biosolve), Methanol (Merck), Standards; CAP (sigma); $^{37}Cl_2$ CAP, 100 mg ampule (RIVM CEC/MAT 09).

The LC conditions included: Column–Symmetry C18 (150×3 mm), 5 µm; Column temperature 30ºC; Injection volume–100 µL; Mobile phase– H_2O /MeOH; Flow–400 µL/min. The MS conditions were: Ionisation mode–APCI, negative; Cone voltage: 20 V; Source block temperature: 120ºC; Desolvation temperature–600ºC; Cone gas–200 L/h; Desolvation gas–550 L/h; CID gas–Argon, p=2.2. 10^{-3} mbar. Methanol (HPLC supra-gradient grade), dichloromethane, ammonia (25%) and toluene (Biosolve (Valkenswaard, The Netherlands) were used. CAP (Sigma–Aldrich, St. Louis, MO, USA) and $^{37}Cl_2$–CAP (RIVM, Bilthoven, The Netherland) were used as reference standards. The standard stock solution was prepared in MeOH at 100 µg/L and stored at -18°C. Dilutions of stock solution were all prepared in Milli–Q H_2O and stored at 4°C. A 0.025% ammonia solution was also prepared using Milli–Q H_2O.

The separation of CAP from the sample components was carried out using LC or by high resolution LC (HRLC). For the QTOF, an X–Bridge C18 analytical column, 3.0×15 mm, 5 µm (Waters) was used to separate CAP in the samples. The LC–system consisted of a vacuum degasser, autosampler and a binary pump (Acquity Waters, Milford, MA). An isocratic mobile phase of ammonia/MeCN (45:55, v/v) was used at a flow rate of 0.4 mL/min and 100 µL injected. For the HRLC, a UPLC (Water, acquity), a BEH C18 analytical column of 2.1×50 mm, 1.7 µm (Waters) placed in a column oven at 50°C were also used. The gradient [solvent A, H_2O (100%); solvent B, MeOH (100%)] was: 0–0.5 min, 10% B; 0.5–3.5 min, linear increase to 100% B, finally held 0.5 min. The analyte (CAP) was eluted after 2.7 min.

For the MS, a Waters Quattro Ultima MS with electrospray ionization (ESI) operating in negative ionization mode was used. The operating parameters were: capillary voltage, 2.7 kV; cone voltage, 25 V; source temperature, 120°C; desolvation temperature, 300°C; cone gas flow, 200 L/h; and desolvation gas, 500 L/h. CAP was fragmented by collision induced dissociation and selected reaction monitoring (SRM) transitions at m/z=321.0>152.1 and m/z=321.0>194.0 were monitored. In the HRLC–MS/MS an additional transition was monitored: m/z=321.0>257.1. $^{37}Cl_2$–CAP was detected by monitoring the transition m/z=324.8>152.0 [10].

2.6.2. Quantification, identification and confirmation, validation

Using matrix match standards curves (with blanks spiked in the range 0 µg/kg–50 µg/kg), CAP in the samples was calculated from the detector response (peak area ratios of the internal standard to CAP standard) and the CAP concentration. LC–MS/MS monitoring two SRM transitions and comparing the SRM–ion ratio from sample and standard was suitable obtaining four identification points [10] in accordance with EU criteria which assures confirmed identity of CAP when a minimum of 4 identification points is earned [17]. In this regard, the LC–MS/MS method used was validated according to guidelines described for quantitative confirmatory methods in Commission Decision 2002/657/EC [17] and reported elsewhere [10].

The plant and soil material were analysed in series each sandwiched by matrix matched calibration standards. For additional selectivity and confirmation purposes the CAP containing samples and some blank samples were reanalysed by very HRLC–MS/MS following EU criteria.

For further confirmation of CAP detected by ELISA and LC–MS/MS, selected grass samples were analysed on UPLC–ToF–MS, using CAP, $C_{11}H_{12}Cl_2N_2O_5$ – 321.0045) and $^{37}Cl_2$–CAP, $C_{11}H_{12}(^{37}Cl_2)N_2O_5$ –324.9986).

2.7.PLANT EXPERIMENTS CONDUCTED IN THE NETHERLANDS

An experiment was conducted in the Netherlands (RIKILT) involving treatment of plants with solutions containing CAP. Four groups of plants were included, each group consisting of eight plants. Blank control (Group A); Blank but possible transfer of a drug (Group B); Treated group low level (Group C); Treated group high level (Group D). Group A was completely separated from the other three groups; Group C separated from the other three groups; while Group B and D were combined. Three treatment solutions were prepared: tap water, 50 µg/L and 1,000 µg/L CAP in water. Where necessary, all plants were treated for two days per week with 50 mL of the solution (or more to ensure the plant survives). Caution was taken to avoid spattering and contamination.

The following scheme was also employed: on week 1 all plants only received water; on week 2 to 25, Groups A and B got water, Group C 50 µg/L CAP and group D 1,000 µg/L CAP. At the end of weeks 1, 9, 17 and 25, two plants on each occasion were selected randomly from each group and separate leaves, roots and stalk obtained if possible. Samples were then stored in a freezer. The samples were then analysed for CAP using an LC–MS/MS.

3. RESULTS AND DISCUSSION

The ELISA test results based on districts and matrix are provided in Table 1.

TABLE 1. ELISA TEST RESULTS

№	Provinces/Districts	Meat types		CAP (µg/kg)		
				1	2	3
1	Zavkhan	Store house	Beef	0.118	0.402	0.25
			Beef	0.164	0.173	0.14
			Beef	0.176	0.13	-
		Bayantes	Beef	-	0.361	0.03
			Goat meat	-	0.226	0.06
			Mutton	-	0.31	0.05
			Horse meat	-	-	-
		Tsagaanchuluut	Beef	0.331	0.308	0.04
			Goat meat	0.05	0.207	-
			Mutton	0.287	0.057	-
			Horse meat	-	-	-
		Urgamal	Beef	-	-	-
			Goat meat	-	-	-
			Mutton	-	-	-
			Horse meat	-	-	-
2	Khuvsgul	Store house	Beef	0.151	0.173	0.21

№	Provinces/Districts	Meat types		CAP (μg/kg)		
				1	2	3
		Ulaan-uul	Beef	0.187	0.179	0.19
			Beef	0.202	0.987	-
			Beef	0.301	0.414	0.46
			Goat meat	0.265	0.104	0.14
			Mutton	0.291	0.356	0.15
			Horse meat	0.371	0.213	0.25
		Jargalant	Beef	0.592	0.284	0.01
			Goat meat	0.212	0.303	0.23
			Mutton	0.356	0.286	0.29
			Horse meat	0.086	0.11	-
		Burentogtoh	Beef	0.219	0.383	0.33
			Goat meat	0.557	0.281	0.20
			Mutton	0.475	0.322	0.26
			Horse meat	-	-	-
		Ulziit	Beef	0.128	-	-
			Goat meat	0.101	-	-
			Mutton	-	-	-
			Horse meat	-	-	-
3	Bayankhongor	Hureemaral	Beef	-	-	0.098
			Goat meat	-	-	-
			Mutton	-	-	0.263
			Horse meat	-	-	-
		Shinejinst	Beef	-	-	-
			Goat meat	-	-	0.118
			Mutton	-	0.075	*
			Horse meat	-	-	-
		Darvi	Beef			-
			Beef	-	-	-
			Goat meat	0.142	-	-
			Mutton	0.207	-	-
			Horse meat	-	-	-
5	Khovd	Bulgan	Beef	-	-	-
			Goat meat	-	-	-
			Mutton	-	-	*
			Horse meat	-	-	*
		Durgun	Beef	-	-	-

№	Provinces/Districts	Meat types		CAP (μg/kg)		
				1	2	3
			Goat meat	-	-	-
			Mutton	-	-	-
			Horse meat	-	-	-
			Beef	-	-	-
		Sagsai	Goat meat	0.062	-	-
			Mutton	1.137	*	*
			Horse meat	-	-	*
			Beef	-	-	-
		Nogoonnuur	Goat meat	0.09	-	-
			Mutton	0.466	0.092	-
			Horse meat	-	-	-
			Beef	-	-	-
		Tolbo	Goat meat	0.192	-	-
			Mutton	-	-	-
			Horse meat	-	-	-
			Beef	-	-	-
		Undurhangai	Goat meat	0.335	0.234	-
			Mutton	0.299	0.297	0.028
			Horse meat	-	-	*
			Beef	-	-	-
7	Uvs	Umnugobi	Goat meat	0.108	0.293	-
			Mutton	0.067	0.306	-
			Horse meat	-	-	-
			Beef	-	-	-
		Malchin	Goat meat	0.048	0.34	0.314
			Mutton	0.318	0.33	0.095
			Horse meat	-	-	-
			Beef	-	-	-
		Delger	Goat meat	-	*	*
			Mutton	-	-	-
			Horse meat	-	-	-
8	Gobi-Altai		Beef	-	-	-
		Khaliun	Goat meat	-	-	-
			Mutton	-	*	*
			Horse meat	-	-	-
		Esunbulag	Beef	-	-	-

№	Provinces/Districts	Meat types	CAP (µg/kg)		
			1	2	3
		Goat meat	-	-	*
		Mutton	-	-	*
		Horse meat	-	-	-

(-): no residue detected; (*): Sample not analysed

Meanwhile of the 2 sets of plants from Mongolia that were analysed using a RIKILT in–house ELISA technique, 0.34 µg/kg to 17.35 µg/kg of CAP was detected in the first batch of 5 and 0.5 µg/kg to 36 µg/kg in the next 10.

3.1.SURVEY AND CAP RESIDUES IN MONGOLIA'S WESTERN REGION

Seventy seven percent of the respondents had good knowledge of bitter plants, 72% had sensed a bitter taste in meat and milk before, 77% knew that bitter tasting plants were common in their vicinity while 76% of the respondents reported a bitter taste in meat and milk in autumn.

Also according to the respondents, the main plants causing bitterness in meat and milk products were most probably *A. macrocephala Jacquem*, *A. Sieversiana Willd*, *A. frigida Willd*, *Th. petaloideum L* and *Th. simplex L*. and most commonly in autumn. These plants were also more dominant among the pasture plants were recent overgrazing and overstocking was reported. Residues of CAP were detected in the range 0.3 µg/kg–3.7 µg/kg, in 11.3% of all samples collected from Khovsgol, Bayan–Ulgii, Bayankhongor, Zavkhan, Khovd and Uvs provinces.

3.2.SURVEY AND CAP RESIDUES IN MONGOLIA'S CENTRAL REGION

On the various commonly used drugs, 35 (96.3%) veterinarians mentioned use of tetracyclines, 31 (81.5%) penicillins, 14 (48.2%) sulphonamides, and 5 (18.5%) aminoglycosides. Meanwhile, no CAP residues were detected in 71 of 81 meat samples from the central region, while the rest had concentration in the range 0.3 µg/kg to 1.47 µg/kg. The CAP residues were detected in 15.94% of sheep meat, 5.45% of goat meat and 18.36% of beef. Generally the residues averaged 0.08 µg/kg in meat of livestock in central region provinces, and specifically 0.01 µg/kg in meat from the Gobi region (Tables 2 and 3).

TABLE 2. SAMPLES IN WHICH CAP RESIDUE WAS DETECTED

No	Sample	No. of samples	Mean, µg/kg	M±m, µg/kg
1	Mutton	11 (15.94%)	0.35	0.35±0.12
2	Goat meat	3 (5.45%)	0.22	0.22±0.07
3	Beef	9 (18.36%)	0.30	0.30± 0.06
4	Total meat	23 (13.29%)	0.31	0.31±0.06

TABLE 3. CAP RESIDUES IN MEAT BY SOURCE

No	Provinces and cities	Livestock species	M	M±m
1	Darkhan-Uul	Beef	0.16	0.16± 0.05
		Mutton	0.11	0.11± 0.05
		Goat meat	0.00	
2	Selenge	Beef	0.13	0.13± 0.08
		Mutton	0.12	0.12± 0.08
		Goat meat	0.15	0.15± 0.06
3	Tuv	Beef	0.07	0.07± 0.03
		Mutton	0.11	0.11± 0.06
		Goat meat	0.04	0.04± 0.02
4	Ulaanbaatar	Beef	0.00	
		Mutton	0.01	0.01± 0.03
		Goat meat	0.00	
5	Gobisumber	Beef	0.01	0.01± 0.008
		Mutton	0.01	0.01± 0.005
		Goat meat	0.02	0.02± 0.01
6	Dornogobi	Beef	0.00	
		Mutton	0.00	
		Goat meat	0.00	
7	Dundgobi	Beef	0.01	0.01± 0.01
		Mutton	0.04	0.04± 0.01
		Goat meat	0.03	0.03± 0.007
8	Umnugobi	Beef	0.00	
		Mutton	0.00	
		Goat meat	0.05	0.05± 0.04

3.3.CAP IN PLANTS AND GEOGRAPHIC DISTRIBUTION OF SUSPECTED PLANT SOURCES

The CAP content in bitter plants ranged from 0.1 µg/kg to 3.9 µg/kg in 29 of the plants. Residues were mainly in Artemisia spp, particularly *A. sieversiana* (0.25 µg/kg to 3.07 µg/kg, mean 1.06 µg/kg) but also *A. frigida* and *Thalictrum simplex*. The levels also varied with region. For example, between 2.5 µg/kg to 3.9 µg/kg levels were detected in *A. frigida* sample collected from Doloon hudag in Emeelt, while *A. frigida* samples from Shaamar, Selenge province, Gachuurt and Bogd mountains, Ulaanbaatar city, and Bayandelger, Tuv province contained 0.01 µg/kg to 0.04 µg/kg. Similar patterns were also observed in other plant species. *A. sieversiana*, *A. anethifolia*, *A. serica*, *A. macrocephala*, and *A. frigida* collected from areas around Khui doloon hudag in Emeelt contained ~1.0 µg/kg of CAP.

The geographic distribution of the suspected bitter tasting plants was evaluated using 8 plant species as indicated below geographical (Table 4).

TABLE 4. DISTRIBUTION OF BITTER PLANTS CHOSEN FOR THE STUDY

Herbs	**Name of geographical counties**															
	Khuvsgul	Khentii	Khangai	Mongolian dagaur	Khyangan	Khovd	Mongol Altai	Middle Khalkha	Eastern Mongol	Great lakes depression	Valley of lakes	Eastern Gobi	Gobi Altai	Zungar gobi	Southern Altai gobi	Alasha gobi
A. macrocephala Jacquem	+	+	+	+		+	+	+	+				+			+
A. Sieversiana	+	+	+	+		+	+	+	+				+			
A. dracunculus	+	+	+	+	+	+	+	+	+				+	+		
A. frigida Willd	+	+	+	+	+	+	+	+	+			+	+			
Th. petaloideum L	+	+	+	+					+							
Th. simplex L	+		+		+	+	+	+	+			+	+			
Iris dichotoma Pall		+						+	+							
Thymus gobicus Tczern	+		+	+			+	+		+	+					

3.4.ANIMAL FEEDING EXPERIMENT

Residues were detected in meat from sheep treated with CAP while none were detected in those fed bitter plants and green hay. No clear explanation could be established for this variation. Other positive results included; meat from sheep treated with CAP and fed hay (3,467 µg/kg); kidney from sheep treated with CAP and fed hay (2,017 µg/kg); liver from sheep treated with CAP and fed hay (2,159 µg/kg); and small intestine from sheep treated with CAP and fed hay (2,858 µg/kg); as well as spleen from sheep treated with CAP and fed with hay (3,195 µg/kg).

3.5.CONFIRMATORY TESTS INCLUDING VALIDATION

As reported elsewhere [10] method accuracy (100% and 104%, respectively) was determined by spiking leaves, roots, soil and stalk material at 0.3 µg/kg and 0.5 µg/kg. The corresponding relative standard deviations were 9% and 6%, respectively, while the method decision limit

(CCα) for plant material was <0.1 µg/kg. The precursor ions for CAP and $^{37}Cl_2$–CAP were (m/z/ 321.9 and 325.9, respectively, while the product ions in both cases were m/z 151±0.5 and 192±0.5 for both and collision energy for both was 15 eV. The same ion transitions were used to identify CAP in blank and spiked samples (Table 5) as well as grass samples (Table 6).

TABLE 5. SELECTED CAP IONS IN BLANK AND SPIKED SAMPLES

Description	**CAP Spike (µg/kg)**	**Selected ions**		
		CAP (m/z 194)	CAP (m/z 152)	CAP IS (m/z 152)
Blank		321>194 1.195e+003	321>152.05 1.960e+003	324.8>152 1.195e+003
Sample 1		321>194 1.443e+006	321>152.05 3.755e+006	324.8>152 3.786e+005
S1 + 0.5 µg/kg	0.5 0	321>194 1.663e+006	321>152.05 4.217e+006	324.8>152 4.348e+005
S1 + 5 µg/kg	5	321>194 2.056e+006	321>152.05 5.165e+006	324.8>152 4.999e+005
S1 + 25 µg/kg	25	321>194 2.570e+006	321>152.05 6.789e+006	324.8>152 6.798e+005
Blank		321>194 1.709e+003	321>152.05 4.901e+003	324.8>152 1.684e+003
Sample 2		321>194 1.190e+006	321>152.05 4.963e+006	324.8>152 4.794e+005
S2 + 0.5 µg/kg	0.5 0	321>194 1.316e+005	321>152.05 3.368e+006	324.8>152 3.426e+005
S2 + 5 µg/kg	5	321>194 8.469e+005	321>152.05 2.135e+006	324.8>152 2.221e+005
S2 + 25 µg/kg	25	321>194 2.047e+006	321>152.05 5.230e+006	324.8>152 5.064e+005
Blank		321>194 2.279e+003	321>152.05 4.277e+003	324.8>152 3.461e+003
Sample 3		321>194 7.247e+006	321>152.05 1.856e+007	324.8>152 1.834e+006
S3 + 0.5 µg/kg	0.5 0	321>194 7.293e+006	321>152.05 1.920e+007	324.8>152 1.803e+006
S3 + 5 µg/kg	5	321>194 5.194e+006	321>152.05 1.327e+007	324.8>152 1.295e+005
S3 + 25 µg/kg	25	321>194 6.739e+006	321>152.05 1.735e+007	324.8>152 1.657e+006
Blank		321>194 4.765e+003	321>152.05 1.361e+004	324.8>152 2.922e+003
Sample 4		321>194 9.080e+005	321>152.05 2.262e+006	324.8>152 4.348e+005
S4 + 0.5 µg/kg	0.5 0	321>194 9.508e+005	321>152.05 2.393e+006	324.8>152 2.590e+005
S4 + 5 µg/kg	5	321>194 2.143e+005	321>152.05 5.434e+005	324.8>152 7.908e+004

Description	CAP Spike (µg/kg)	Selected ions		
		CAP (m/z 194)	CAP (m/z 152)	CAP IS (m/z 152)
S4 + 25 µg/kg	25	321>194 2.132e+005	321>152.05 5.600e+005	324.8>152 8.665e+004
Blank		321>194 1.696e+003	321>152.05 3.716e+003	324.8>152 1.699e+003
Sample 5		321>194 1.598e+004	321>152.05 3.994e+004	324.8>152 3.458e+004
S5 + 0.5 µg/kg	0.5 0	321>194 6.691e+003	321>152.05 1.778e+004	324.8>152 3.608e+004
S5 + 5 µg/kg	5	321>194 1.416e+004	321>152.05 3.926e+004	324.8>152 3.707e+004
S5 + 25 µg/kg	25	321>194 1.766e+004	321>152.05 4.649e+004	324.8>152 3.501e+004

TABLE 6. ANALYSIS OF CAP IN GRASS SAMPLES BY UPLC–TOF–MS

Sample	Spike level (µg/kg)	Weight (g)	Spike CAP (50 (µg/L) (µL)	IS(50 µg/L) (µL)	H_2O (mL)
Sample 1	0	1	0	40	20
S1 + 0.5µg/kg	0.5	1	10	40	20
S1 + 5 µg/kg	5	1	100	40	20
S1 + 25 µg/kg	25	1	500	40	20
Sample 2	0	1	0	40	20
S2 + 0.5 µg/kg	0.5	1	10	40	20
S2 + 5 µg/kg	5	1	100	40	20
S2 + 25 µg/kg	25	1	500	40	20
Sample 3	0	1	0	40	20
S3 + 0.5 µg/kg	0.5	1	10	40	20
S3 + 5 µg/kg	5	1	100	40	20
S3 + 25 µg/kg	25	1	500	40	20
Sample 4	0	1	0	40	20
S4 + 0.5 µg/kg	0.5	1	10	40	20
S4 + 5 µg/kg	5	1	100	40	20
S4 + 25 µg/kg	25	1	500	40	20
Sample 5	0	1	0	40	20
S5 + 0.25µg/kg	0.5	1	5	40	20
S5 + 0.5 µg/kg	5	1	10	40	20
S5 + 1 µg/kg	25	1	20	40	20

Up to 450 µg/kg of CAP was detected in plant samples obtained from Mongolia, a local store in the Netherland, as well as a sample of *A. fridiga* from Utah, USA. Also in Mongolian sample sets of leaves, roots, stalk of *A. sieversiana, A. frigida* as well as green grass, soil and water samples (n=192), CAP residue concentrations ≥0.1 µg/kg were detected in 15% of the analysed samples. Some 5 samples contained CAP between 1 µg/kg and 5 µg/kg. Also, a number of root samples also contained CAP. No specific relationship was found between the concentration of CAP and specific location [10].

Using EU criteria [17] two representative non–compliant (CAP containing samples were analysed by LC–MS/MS [10] were evaluated. With reference to the control samples an average ion ratio of 39.5% and a relative retention time (RRT) of 1.008 (acceptable relative retention time being 2.5%) were determined. Since the maximum allowed relative deviation of the ion ratio of 25%, the identity of CAP was confirmed if the ion ratio is between 29.5% and 49.1% [10].

Using a blank herb mixture sample, a blank herb mixture sample fortified with 2 µg/kg CAP, and a non–compliant herb mixture sample containing 4 µg/kg, method selectivity was evaluated following analysis on an HRLC–MS/MS system. Ion ratios meeting the EU requirements [17] were obtained [10]: from the control samples an average ion ratio of 37.6% calculated for the product ions m/z=194/152 and 70.2% for m/z=257/152, as well as relative retention time (RRT) of 1.004 was calculated.

Also based on EU criteria and reported elsewhere [14] where the maximum allowed relative deviation of the ion ratio of 25% is required for ion ratios m/z=194/152, and 20% for m/z=257/152, CAP was confirmed in the noncompliant samples since the relative deviations compared to the control sample were from -1.3 to -16.4% for m/z=194/ 152 and -1.1% to -5.1% m/z=257/152 (thus within limits). The samples and control also had the same retention time of 1.004 min [10]. The identity of CAP was therefore unambiguously confirmed. The LC results were in agreement with those from the HRLC.

Thus the LC–MS/MS confirmatory analyses of plant materials (belonging to different families) from Mongolian pastures, Utah, USA and local store in the Netherlands had CAP with levels in some cases far exceeding EU criteria/guidelines.

The present study confirmed high concentrations of CAP was detected in several bitter plants such as *A. sieversiana* and *Thalictrum simplex* by using both ELISA and HPLC techniques, and the results were confirmed in a Dutch drug residue laboratory. Screening tests for this antibiotic were performed in small intestines collected by export oriented enterprises, and meat of livestock in both central and western regions of Mongolia.

3.6.PLANT EXPERIMENTS IN NETHERLANDS

Samples in the treatment experiment were analysed in the Netherlands for CAP AND Very low levels (≤0.5 ng/g plant material) detected. Using an LC–MS/MS method in Belfast, *A. macrocephalo* and *Th. simplex* were found contain CAP levels above 100 ng/kg and *Th. Simplex* less than 1 ng/kg, respectively.

4. CONCLUSION

Although CAP was detected in some bitter pasture plants, including *A. sieversiana*, *Th. simplex* and *A. frigida*, and green grasses, the levels depend more on geographical location than the species. The herbs growing on the Mongolian pastures do not always contain high

concentrations of CAP and no single herb family appears to be responsible for the bioaccumulation of this antimicrobial agent. Also a large number of root samples contained CAP. A research carried out in western and central regions showed CAP in pasture plants grazed on by livestock. Small intestines of Mongolian sheep contained 0.3 µg/kg to 3.7 µg/kg of CAP. Residues were detected in pasture plants with bitter taste by ELISA, LC–MS/MS and UPLC–ToF–MS. The latter 2 tools where used for confirmation.

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APPLICATION OF AN ISOTOPE DILUTION METHOD TO INVESTIGATE THE POSSIBLE NATURAL OCCURRENCE OF CHLORAMPHENICOL IN POULTRY LITTER

KANARAT, S[*]., TANGSIRISUP, N[*]., NIJTHAVORN, N[*].,ELLIOTT, C[**]., CANNAVAN, A.,[***]
[*] Veterinary Public Health Laboratory (VPHL), Department of Livestock Development, Bangkok, Thailand
[**] Institute of Agri–food and Land Use, Queens University Belfast, Belfast, UK
[***] International Atomic Energy Agency

Abstract

In recent years the detection of residues of chloramphenicol (CAP) in foods such as poultry and honey has had a major impact on international trade. Follow–up investigations in Thailand relating to poultry non–compliant findings in Europe have, in some cases, been unable to establish the cause of the residues, since there was no recent history of use of the drug. A possible source of contamination is the biosynthesis of CAP by *Streptomyces venezuelae* or other actinomycetes. A study was instigated to investigate this possibility in a typical poultry production environment. A sensitive and specific liquid chromatography–tandem mass spectrometry (LC–MS/MS) isotope dilution method was used to detect and quantify low levels of CAP in the experimental samples. *S. venezuelae* in CAP–producing phase was spiked into poultry litter under various conditions and the litter was tested for growth of the organism and for CAP concentration. Results showed that *S. venezuelae* was not viable after 3–4 weeks and initial levels of CAP from the *S. venezuelae*/normal saline solution added (maximum 0.6 µg/kg) decreased rapidly and were below the limit of quantification (LOQ) of the analytical method (0.04 µg/kg) by week 3. Litter samples collected from 5 poultry farms with a history of poultry contamination with CAP were tested and found to be negative for both CAP and *S. venezuelae*. The results suggest that residues of CAP on the farms tested were extremely unlikely to have been caused by natural biosynthesis of the drug in the production environment.

1. INTRODUCTION

The drug CAP is a broad spectrum antibiotic used in both human and veterinary medicine. It is biosynthesised by the soil organism *S. venezuelae* and several other actinomycetes, but is produced for commercial use by chemical synthesis [1]. The drug has been evaluated by the Joint FAO/WHO Expert Committee on Food Additives, most recently at its sixty–second meeting [2], by the European Committee for Veterinary Medicinal Products [3] and by a number of other agencies. Because of concerns over its genotoxicity, embryotoxicity and fetotoxicity, carcinogenic potential and the lack of a dose–response relationship for aplastic anaemia caused by treatment with CAP in humans, the drug has been banned for use in food–producing animals in the European Union (EU) and in many other countries.

However, because of its efficacy and cost–effectiveness, CAP continues to be used in food animals in some countries, especially in the developing world, as evidenced by sporadic reports in the EU Rapid Alert System for Food and Feed. Because of the detection of CAP and other antimicrobials such as nitrofurans, the European Commission (EC) adopted a series of Decisions (2001/699/EC, 2001/705/EC, 2002/251/EC) requiring testing of products for export to the EU from various countries in East Asia and Latin America to protect the European consumer from imported aquaculture and poultry products containing potentially harmful residues of these substances. A minimum required performance limit (MRPL) of 0.3 µg/kg was assigned by the EC in 2003 [4] for analytical methods to detect CAP.

Sporadic non–compliant results continue to arise in products from various countries even after measures have been put in place to control the use of CAP. Various hypotheses have been suggested to explain these results. Residues may be caused by the use of unlicensed and/or counterfeit pharmaceutical products containing undeclared CAP [5]. Other possibilities include persistent environmental contamination resulting from past use of the drug,

contamination from topical medicinal preparations containing CAP used by processing workers, and ingestion of naturally occurring CAP from the environment [6]. This last possibility was suggested as an explanation for contaminated products originating from several poultry producers in Thailand where there was no evidence of the recent use of CAP. Any evidence of natural or environmental sources of CAP contamination would have serious implications for the EU's 'zero tolerance' policy within the Union and the application of the MRPL as an action level for imports.

A study was designed and carried out at the Veterinary Public Health Laboratory in Bangkok to test the natural–production hypothesis by introducing *S. venezuelae* to chicken litter under conditions prevalent in the production facilities of the affected farms and testing for viability of the organism and production of CAP. Litter samples from producers with a history of CAP–contaminated poultry were also tested for the presence of *S. venezuelae* and CAP. To detect and determine the concentration of CAP in the experimental samples, a highly specific and sensitive LC–MS/MS method was applied, which used deuterated CAP as internal standard (IS) to quantify CAP present by isotope dilution. The method was adapted from a method developed in the VPHL to determine CAP in poultry meat for regulatory purposes.

2. MATERIALS AND METHODS

2.1. STABILITY OF *S. VENEZUELAE* IN NORMAL SALINE

This study was performed to ensure that *S. venezuelae* could survive in normal saline, since a suspension in normal saline would be used in the litter experiments. *S. venezuelae*, strain DSM 40232, was streaked onto GYM *Streptomyces* agar and incubated at 28°C for 48 h. Several colonies were harvested and suspended in sterilized normal saline. The suspension was well mixed and centrifuged to obtain a pellet. The procedure was repeated, normal saline added to the combined pellets and the suspension was mixed and stored at room temperature. *S. venezuelae* was enumerated daily for 1 week (trial 1) and weekly for 3 weeks (trial 2).

2.2. MOISTURE CONTENT OF LITTER FROM POULTRY PRODUCERS

Twenty samples of litter were collected from broiler farms at the broiler ages of 1 day, and 1, 2, 3, 4, 5 and 6 weeks and analysed for moisture content using ISO method 1442. Samples were also examined for the presence of *S. venezuelae*.

2.3. STABILITY OF CAP IN LITTER

The stability of CAP in litter was investigated With portions of litter (20 g) weighed into 11 bags and CAP solution (1 ng/mL, 28 mL) added to 10 of the bags before the contents were mixed well. The 10 spiked bags were pooled and again mixed well to homogenize the CAP distribution. The pooled sample was once more divided into 20 g portions, stored in 10 bags at room temperature along with the control (unspiked) bag and assayed for CAP weekly.

2.4. CAPABILITY OF *S. VENEZUELAE* TO PRODUCE CAP IN LITTER.

An experiment to investigate capability of *S. venezuelae* to produce CAP in litter was designed. Portions of litter (20 g) were weighed into 21 bags. *S. venezualae* suspension (1.0×10^8 org/mL) was added, 10 mL to bags 1–10 and 20 mL to bags 11–20. The contents were mixed well. The contents of bags 1–5, 6–10, 11–15 and 16–20, respectively, were pooled to produce 4 samples. Each sample was mixed well to ensure homogeneity. Both the suspension and the spiked litter were stored at room temperature and assayed for CAP weekly

for 5 weeks. *S. venezuelae* was also enumerated weekly in both the spiked litter samples and the suspension.

2.5. VIABILITY OF *S. VENEZUELAE* IN LITTER WITH DIFFERING MOISTURE CONTENT

S. venezuelae – normal saline suspension was added to sterile litter with ratios of litter to *S. venezuelae* suspension of 1:1, 1:0.5, and 1:0.25 (w/v, g:mL). Each spiked sample was mixed well and analysed for moisture and for presence of *S. venezuelae* weekly for 6 weeks.

2.6. DETERMINATION OF CAP

The CAP level was determined in chicken litter using an LC–MS/MS method developed and validated at the VPHL, Bangkok. Briefly, a portion of well homogenised litter was extracted by sonication with phosphate buffered saline, sodium chloride and MeCN. The extract was washed with hexane and extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness under a stream of nitrogen, re–dissolved in H_2O and cleaned up by C18–SPE. The methanol (MeOH) eluate was evaporated to dryness under a stream of nitrogen and re–dissolved in MeOH/H_2O (1:1, v/v) for analysis by LC–MS/MS. Multiple reaction monitoring was employed for the transition ions at m/z 321–257, 321–152 and 326–157 (deuterated internal standard). Quantitation was by the isotope dilution method using the ratios of the transitions at m/z 321–152 and mz/ 326–157.

2.7. LITTER SAMPLES FROM FARMS WITH A HISTORY OF CAP DETECTION IN POULTRY

Samples of litter were taken from 5 broiler farms with a recent history of poultry meat containing residues of CAP. The samples were tested for the presence of *S. venezuelae* and assayed for CAP.

3. RESULTS AND DISCUSSION

3.1. STABILITY OF *S. VENEZUELAE* IN NORMAL SALINE

The results of the first experiment in this study showed that *S. venezuelae* was viable for at least 7 days in normal saline, but the cell count dropped by more than 95% after 2–3 weeks. A suspension in normal saline could therefore be used up to a few days after preparation.

3.2. MOISTURE CONTENT OF LITTER FROM POULTRY PRODUCERS

The average moisture content of the litter samples taken from broiler producers almost doubled over the 6 week period of the investigation, from about 20% on day 0 to about 38% after 6 weeks. No *S. venezuelae* cells were detected in any of the samples taken.

3.3. STABILITY OF CAP IN LITTER.

The mean CAP concentration in the 10 litter samples spiked with CAP and prepared as described above decreased gradually from 0.60 μg/kg to 0.37 μg/kg over 7 weeks.

3.4. CAPABILITY OF *S. VENEZUELAE* TO PRODUCE CAP IN LITTER

The capability of *S. venezuelae* to produce CAP in litter was investigated by spiking litter samples with suspensions of the organism in CAP–producing phase, as described above. Although the CAP concentration in the normal saline suspension remained constant (8.3±0.83 μg/kg, mean ± SD) over the 5 week period of the trial, the concentrations in the 4 litter samples spiked with the *S. venezuelae* suspension decreased from initial measured concentrations (0.26 μg/kg–0.6 μg/kg) to less than the detection limit of the method after 3 weeks.

3.5. VIABILITY OF *S. VENEZUELAE* IN LITTER WITH DIFFERING MOISTURE CONTENT

The investigation into the viability of *S. venezuelae* in litter with differing moisture content demonstrated that there the organism was not viable after 3–4 weeks in litter with approximately 30%, 40% or 50% moisture.

3.6. DETERMINATION OF CAP

The chromatograms were free from interference at the retention time of CAP. Quantitation was by the isotope dilution method using the ratios of the transitions at m/z 321–152 and m/z 326–157 (deuterated internal standard). The method performance characteristics were evaluated and the method was shown to be fit–for–purpose. The LOQ, by isotope dilution, was ~ 0.04 μg/kg.

3.7. LITTER SAMPLES FROM FARMS WITH A HISTORY OF CAP DETECTION IN POULTRY

No *S. venezuelae* organisms were isolated from the samples of litter taken from 5 broiler farms with a recent history of poultry meat containing residues of CAP. No CAP was detected in the samples.

The experimental design for this study was guided by preliminary trials in which litter samples were spiked with *S. venezuelae* in GYM *Streptomyces* broth. The preliminary results indicated that there may have been some limited production of CAP in the poultry litter. However, it was suspected that the CAP detected was actually contained in the broth used to spike the samples, in which the *S. venezuelae* had been incubated for 48 h. It was also likely that the GYM broth added to the litter was able to support the growth of the organism whereas the litter alone, without the nutrients contained in the broth, might not support growth. The study was designed, therefore, to use normal saline as the spiking medium. The results of the first experiment in this study showed that *S. venezuelae* was viable for at least 7 days in normal saline, but the cell count dropped by more than 95% after 2–3 weeks. A suspension in normal saline could therefore be used up to a few days after preparation.

It was further suggested by preliminary studies that CAP, which was present initially at easily measurable concentrations, was unstable in poultry litter, since no CAP was detected after 3 weeks. However, the results of the present study showed that the mean CAP concentration in the 10 litter samples spiked with CAP decreased gradually from 0.60 μg/kg to 0.37 μg/kg over 7 weeks.

The capability of *S. venezuelae* to produce CAP in litter was investigated by spiking litter samples with suspensions of the organism in CAP–producing phase, as described above.

Although the CAP concentration in the normal saline suspension remained constant (8.3 ± 0.83 µg/kg, mean ± SD) over the 5 week period of the trial, the concentrations in the 4 litter samples spiked with the *S. venezuelae* suspension decreased from initial measured concentrations (0.26 µg/kg–0.6 µg/kg) to less than the detection limit of the method after 3 weeks.

In the experiment described above to investigate the stability of CAP in litter spiked with the drug, CAP concentrations, initially in the same range as those in the *S venezuelae* spiked samples, remained easily measurable for at least 7 weeks. The rapid decrease in concentration in litter spiked with *S. venezuelae* may be due to the deactivation and/or degradation of CAP by the organisms. It has been shown previously that cultures of *S. venezuelae* not engaged in CAP synthesis are strongly inhibited if exposed to the antibiotic, but become resistant by inactivating it.

It was postulated that the moisture content of the litter may be an important factor in its feasibility as a growth medium for *S. venezuelae*. The experimental protocol affected the moisture content of the litter used in the study, since the *S. venezuelae* was added as a suspension in normal saline. It is possible that litter with higher moisture content may provide a more suitable medium for the anaerobic growth of the organisms. Many factors affect both the growth of *S. venezuelae* and the biosynthesis of CAP by the organism. For example, the biosynthesis of CAP is usually uniphasic, linked with vegetative growth of the bacterium, but can also be biphasic and not linked with biomass accumulation, depending on composition and accessibility of available nutrients [1, 7]. To gain some knowledge of the moisture content, 20 samples of litter from working broiler producers were collected at various time points over the broiler production cycle from day 0 to week 6. The average moisture content almost doubled, from about 20% on day 0 to about 38% after 6 weeks. The investigation into the viability of *S. venezuelae* in litter with differing moisture content demonstrated that there was no growth of the organism after 3–4 weeks in litter with approximately 30%, 40% or 50% moisture.

The rapid decrease in CAP concentration and the apparent lack of viability of *S. venezuelae* in the litter may have been at least partly due to inhibition of the growth of *S. venezuelae* after its addition to the litter by the CAP produced in the starter culture conditions in GYM broth and/or in normal saline, and the simultaneous deactivation of the CAP by the *S. venezuelae* which was no longer in an environment that supported CAP biosynthesis.

The samples of litter collected from 5 farms with a recent history of CAP residues in poultry meat contained no detectable CAP and *S. venezuelae* was not isolated from any of the samples. This result suggests that the non–compliant results for poultry from these producers arose neither from natural CAP synthesis nor from persisting environmental residues of CAP resulting from historical veterinary uses.

4. CONCLUSION

The results of this study strongly suggest that CAP–contaminated poultry tissue samples originating from producers in Thailand were not caused by natural occurrence of CAP in the production environment. This is an important finding in relation to the continuance of the enforcement of the EU MRPL for CAP.

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DEVELOPMENT OF MICROBIOLOGICAL SCREENING METHOD FOR THE DETECTION OF MULTI–RESIDUES OF ANTIMICROBIAL SUBSTANCES

KANARAT, S.,

Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand

Abstract

Suitable bacterial strains, optimal media for each stain, and respective concentration for the detection of Chlortetracycline (CTC), Doxycycline (DOX), Penicillin (PEN), Erythromycin (ERY), Tylosin (TYL), Trimethoprim (TMP), Ciprofocaxin (CIP), Enrofloxacin (ENR) and Norfloxacin (NOR) were studied.

Commonly used extraction solutions such as acetonitrile (only for eggs) were also tested for possible growth inhibition of selected bacteria in the relevant media so as to avoid false positive results. No negative effect was observed. Matrix effects on the microorganisms under study were also tested. Milk and meat showed no matrix effects unlike honey, egg white, and serum that showed inhibitory effects. Consequently, honey was diluted with phosphate buffer to overcome the inhibitory effect while use of egg white was omitted altogether. In case of serum, only the MHA7.2 + *Gebacillus Stearothermophilus* plate was excluded being the only one that showed inhibitory effect with serum. Serum, honey, milk and egg yolk samples were directly applied to the plates to avoid dilution and to optimize sensitivity. Honey was diluted in a 1:1 ratio with phosphate buffer before application on the plates.

The prototype test was developed and the sensitivity compared with a standard UK 6–plate method. The UK 6 plate method was more sensitive than the developed method. Some antibiotics were detected at twice the maximum recommended residue limits (MRL). Improving detection at the MRL level for better method performance required sample concentration which in itself is laborious.

The study also noted that non–extracted/purified samples (such as meat) were associated with more sensitive results compared to extracted samples. This observation guides development of a prototype assay for routine detection of multi–residue antimicrobial substances in various matrices.

1. INTRODUCTION

The affordability, reliability and practicability of a microbiological screening method for the detection of antimicrobial residues, enhances food safety programs in developing countries.

Previous studies have shown how the following conditions enhance method sensitive: The most optimal media for each bacterial strain being: Antibiotic medium no. 1 (Oxoid) adjust pH to pH 6, Mueller Hinton Agar (MHA) (Oxoid) adjust pH to pH 7.2 and pH 8, Plate Count agar (PCA) (Oxoid) adjust pH to pH 8, Test Agar pH 6 (Merck). The most suitable bacterial strains being *Bacillus cereus* ATCC 11779, *B. megaterium ATCC 9885, G. stearothermophilus* ATCC 10151, *Escherichia coli* ATCC 11303, *Kocuria rhizophila* ATCC 9341. Meanwhile the recommended suitable concentration of bacterial strain was $2x10^6$ cells/mL by using 2 mL bacterial strain suspension per 200 mL designated agar and that using agar plate containing 2×10^4 cells/mL of *B. megaterium, B. cereus, G.stearothermophilus, K.rhizophila* and *E.coli*, the method could detect CTC, DOX, PEN, ERY, TYL, TMP, CIP, ENR and NOR (prepared in solution) at 0.5 MRL; Tilmicocin (TIL) and Neomycin (NEO) at 1 MRL and Oxytetracycline (OTC) at 2 MRLs based on Codex [1] and European Union [2] standards. Furthermore, the preparation of agar plate for the experiment would include: adding 2 mL of $2x10^6$ cells/mL of each bacterial strain to 200 mL of the agar mentioned above, pouring 5 mL of the agar added with the bacterial strains into sterile plate. For the agar added with *G. stearothermophilus*, 15 mL would be poured into the plate. These would be kept ate 3ºC $\pm$2ºC.

The objective of the study was to develop a microbiological screening method for the detection of antimicrobial residues that is inexpensive, reliable, practicable and affordable to developing countries in order to enable these countries to start establishing simple residue monitoring plan. Work included the study on sample extraction for different matrices including meat, milk, serum, honey and eggs and production of a prototype assay for routine use with a selection of matrices including sample preparation procedures.

2. MATERIALS AND METHODS

In the current study, preparation of media (Annex I), bacterial suspension (Annex II), and agar plates (Annex III) were investigated.

Following literature review, the following were selected as extraction solutions for meat, honey, milk and serum. Acetonitrile (MeCN), Methanol (MeOH), MeCN + MeOH, 19:1 (v/v), Phosphate buffer, Acetone + H_2O (2:1) and no extraction solution. Matrices were placed directly onto the prepared agar plate.

2.1. TESTING THE INHIBITION EFFECT OF THE EXTRACTION SOLUTION ON THE GROWTH OF MICROORGANISMS.

Potential inhibitory effect of selected extraction solutions on the growth of bacterial strains (which potentially leads to false positive results) was investigated following the procedure below.

(1) Pipetting 5 mL MeCN to 5 g of ground chicken meat.
(2) Homogenizing the mixture well.
(3) Letting the homogeneous mixture to precipitate.
(4) Dipping 6–mm. diameter filter paper into the liquid part of the mixture.
(5) Placing the filter disc onto prepared agar plate indicated in Annex III.
(6) Incubating the plate overnight at the appropriate temperature indicated in Annex III.
(7) Interpreting the result.
(8) Repeating steps 1–7 but use extraction solutions other than MeCN.

Observation
There were no inhibition zones (clear zone) around the disc. This indicated that the extraction solution selected for this study did not inhibit the growth of the microorganism under study.

2.2. TESTING FOR MATRIX EFFECT ON THE MICRO–ORGANISM UNDER STUDY.

It is necessary to ensure that the matrix under study has no inhibition effect to the growth of the microorganism which is used as the indicator for antimicrobial residue detection. In principle if a sample contains antimicrobial substances, the growth of the microorganism will be inhibited leading to inhibition zone (clear zone) around the sample. However, if the matrix has inherent inhibition effect on the growth of the microorganism used, a zone of inhibition would results around the sample leading to false positive results.

2.2.1. Meat sample (chicken meat, beef and pork)

The procedure involved the following
(1) Punching frozen meat sample with sterile cock borer with diameter 6 mm.
(2) Pushing the punched meat out and cuting it in 2 mm. thickness with sterile scissor.
(3) Placing the meat on every agar plate prepared according to Annex III.

(4) Keeping the plate at room temperature for 1 h.
(5) Incubating each plate overnight at the appropriate temperature indicated in Annex III.
(6) Interpreting the result.

Observation
There was no zone of inhibition (clear zone) around the meat sample meaning the meat had no inhibitory effect on the growth of microorganisms used in this study.

2.2.2. Milk

The following procedure was followed

(1) Punching the agar in agar plates using an 8 mm in diameter sterile cock borer, and discharging the agar by sucking or using sterile forceps or inoculating loop.
(2) Pipetting 10 µm of milk sample and inoculating into the hole in each agar plate.
(3) Keeping the plate at room temperature for 1 h.
(4) Incubating each plate overnight at the appropriate temperature as indicated in Annex III.
(5) Interpreting the results by measuring the zone of inhibition (clear zone) from the edge of complete clear zone against the lawn of bacterial growth at one side and ending at the edge at the opposite side of each inoculated hole. The diameter was measured twice, the second measurement being perpendicular to the first, and the means calculated.

Observation

The milk had no inhibition effect on the growth of the microorganism used in this study.

2.2.3. Honey

In this procedure, the honey was diluted with phosphate buffer saline solution (PBS) to 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8 and 1:9 (w/v). The steps (1, 2, 3, 4 and 5) used for the milk samples was repeate dor the honey samples although using undiluted (1:0) and diluted honey instead of milk.

Observation
There were inhibition zones around the holes containing undiluted sample on every prepared agar plate. For diluted honey, there were no inhibition zones on every plate.

Conclusion
In order to avoid matrix inhibition effect, honey sample should be diluted with phosphate buffer to 1:1 (w/v) before applying it onto agar plate.

2.2.4. Chicken egg, Egg yolk, Egg white, Egg yolk and egg white mixture

As for the honey, the steps 1, 2, 3, 4 and 5 used for the milk samples were followed but using egg yolk, egg white and mixture of egg yolk and egg white instead.

Observation
Egg yolk: No inhibition zones showed around the hole containing sample tested except the agar plate containing MHA 7.2 + *G. stearothermophilus.*
Mixture of egg yolk and egg white: There were zone of inhibition around the samples in all plates except the samples tested on AB_6 + *Kocuria rhizophila* MHA_8 + *K. rhizophila* and MH_8 + *E. coli.*
Egg white: There were zones of inhibition around the sample tested in every plate.

Conclusion
By using the current, egg yolk did not inhibit the growth of the microorganisms under study, but egg white and mixture of egg white and egg yolk had inhibition effect on the growth of the microorganisms under study. Therefore, egg yolk was the best matrix to represent egg samples and only thus egg yolk will be used for conducting this test. However, it was obvious that egg yolk samples also had inhibition effect when conducted the test on MHA 7.2+*G. stearothermophilus* plate leading to a false positive result. Therefore, to avoid false positive when the samples are egg yolk, this plate should not be included in the test.

2.2.5. Serum sample

As for the honey and egg samples, to the steps 1, 2, 3, 4 and 5 used in preparing the milk samples were repeated but using serum sample instead.

Observation
There were no zones of inhibition occurring around the hole containing the sample except the sample tested on the MHA 7.2 + *G. stearothermophilus*.

Conclusion
To avoid false positive result, the MHA7.2 + *G. Stearothermophilus* plates would not be used for serum sample.

2.3. STUDY ON THE APPROPRIATE EXTRACTION SOLUTION AND SAMPLE PREPARATION PROCEDURE

2.3.1. Serum, honey, milk and egg yolk samples

The principle of the method under study was that any antimicrobial substances in the sample will diffuse into agar and inhibit the growth of the microorganism used in this method. Therefore, if extraction solution is added to a sample, the sample would be diluted lowering method sensitivity. Thus, liquid samples including egg yolk, serum, and milk were not be extracted, rather applied to the prepared agar plates directly. However, since undiluted honey itself has inherent inhibitory effects on the microorganisms used in every plate, it was necessary to dilute the honey sample with phosphate buffer solution at the ratio of 1:1 (w/v) to prevent false positive result.

2.3.2. Meat Sample

The procedure for the meat samples followed the following

(1) Dividing meat sample into 3 portions; each portion weighing at least 100 g.
(2) Spiking the meat samples with a solution of antimicrobials at the concentration of 0.5, 1 and 2 MRL [1, 2], and homogenizing each sample very well.
(3) Freezing the sample overnight.
(4) Punching each frozen meat portion with a sterile 8 mm diameter cork borer.
(5) Pushing the punched meat out and cutting the meat with sterile scissors to obtain 14 pieces (2 mm thick).
(6) Placing each piece of meat from the 3 portions onto each prepared plates for each antimicrobial.
(7) Keeping the plates at room temperature for 1 h.
(8) Weighing the rest of each 5 g portion unless 5 subsamples are needed from each portion.
(9) Adding 5 mL of MeCN, 5 mL MeOH, 5 mL MeCN + MeOH (19:1, v /v), 5 mL MeCN + H_2O (2:1) and 10 mL phosphate buffer to each subsample of each set (0.5, 1 and 2 MRLs).
(10) Homogenizing each subsample well.
(11) Dipping 14 filter paper with 6 mm in diameter to each subsample and waiting until the filter paper absorbs the mixture solution thoroughly.
(12) Placing the absorbed filter paper onto the surface of agar plate for each antimicrobial (2 disc/agar plate)
(13) Keeping the plate at room temperature for 1 h.
(14) Incubating the plate overnight at the appropriate temperature according to Annex III.
(15) Reading the test plates by measuring the zone of inhibition (clear zone) from the edge of complete clear zone against the lawn of bacterial growth at one side and ending at the edge at the opposite side of each meat disk. Measurement at the diameter wwas conducted twice, the second measurement perpendicular to the first. Compute the means of the 2 measurements.

Observation

The study showed that the meat sample without extraction gave more sensitive result than extracted meat. So the meat sample will be applied directly onto the plate after punching with 8 mm diameter cork–borer and cut into 2 mm thick.

Conclusion

Unextracted meat sample gave more sensitive result than extracted meat. So meat sample will be used directly without extraction in this study.

2.4. PREPARATION OF BACTERIAL SUSPENSION MEDIA AND AGAR PLATES

Seven types of agar plates (Annex III) were used namely:

(1) Mueller Hinton Agar, pH8 (MHA8) + *Kucoria rhizophila*
(2) Test agar, pH6 (CM6) + *G. stearothermophillus*
(3) Plate Count Agar, pH8 (PCA8) + *K. rhizophila*
(4) Antibiotic Medium No.1, pH6 (AB6) + *B. cereus*

(5) Mueller Hinton Agar, pH7.2 (MHA7.2) + *B. megaterium*
(6) Mueller Hinton Agar, pH7.2 (MHA7.2) + *G. sterrothemophillus*
(7) Mueller Hinton Agar pH8 (MHA8) + *E. coli*

Preparation of agar bacterial suspension and gar plates is shown in Annex I, II and III (Tables I–1 and I–2) while the preparation of antimicrobial solutions is detailed in Annex IV.

2.5. PREPARATION OF SAMPLES

For milk and serum samples, no sample preparation is needed and for the egg sample, aseptically separate egg yolk from egg white. Honey samples were weighed (1 g) and pipette 1 mL of phosphate buffer added before mixing homogeneously. For the meat sample, frozen samples were punched with a sterile 8 mm diameter cork borer and a sterile scissor used to cut the meat in 2 mm thickness to obtain 17 pieces. Samples were then kept the refrigerator and conduct the test sample as soon as possible.

2.6. PROCEDURE

2.6.1. Testing of tissue samples

Empty and sterile plates were prepared for keeping sliced tissue samples by marking ten (x) marks on the bottom of the plate. One plate could keep 10 samples number; plate numbers were labelled as 0, 1, 2, 3 and the lids/bottoms marked at the position where the first sample is placed. Frozen tissue samples were thawed at room temperature for up to 20 min and a cylindrical core cut from each tissue using the cork borer, and the frozen core discharge using a sterilized metal pusher.

In total 14 slices of each sample (2 mm thickness) were cut using sterile scissors or scalpels. Both end slices and any slices containing fat and/or skin were discarded. Scissors and scalpels were sterilized between samples by cleaning thoroughly and soaking in methylated spirit and flamed. The slices were then placed on the (x) mark in clockwise direction on each plate. Samples in duplicate were set at room temperature for ~30 min before incubateing as appropriate.

2.6.2. Testing of eggs and serum

The egg yolk and white were aseptically separated before discarding the white. Using a fresh pipette tip for every sample 2 wells were filled with yolk and serum on each of the six plates for each sample. This was not conducted on the plate containing MHA7.2 + *G. stearothermophilus*.

2.6.3. Testing of honey and milk

Phosphate buffer (1 mL) was added to 1 g honey and mixed well. Using a sterile pipette tip for every sample, 2 wells were filled with diluted honey and milk on each of the 7 plates for each sample.

2.6.4. Antimicrobial control discs

A sterile blank disc is placed at the middle of each plate. Pipette 10 µL of the prepared working standard solutions and slowly drop onto each blank disc [Table 1].

TABLE 1. TEST PLATE, ANTIMICROBIAL CONTROL DISC, MINIMUM ZONE SIZE, TARGET ZONE SIZE AND SYMBOL OF THE TEST PLATE

Test plate	Antimicrobial control disc	Minimum zone size	Target zone size
CM 6 + *G. stearothermophilus* MHA 7.2 + *G. stearothermophilus*	PEN G (0.01 Unit/disc)	24 mm	30 mm
MHA 7.2 + *B. megaterium*	Sulphadimidine (SDM, 0.5 µg/disc)	20 mm	30 mm
PCA 8 + *K. rhizophila* MHA 8 + *K. rhizophila*	ERY (0.025 µg/disc)	24 mm	29 mm
AB 6 + *B. cereus*	CTC (0.5 µg/disc)	28 mm	32 mm
MHA 8 + *E. coli*	CIP (0.003 µg/disc)	28 mm	32 mm

2.6.5. Incubation and reading of test plates

The test plates were incubated at the appropriate temperature for 18–24 h. Zones of inhibition due to the samples or control discs were measured to the nearest millimetre starting from the edge of complete clear zone against the lawn of bacterial growth at one side and ending at the edge at the opposite side. The zone reader was used as a standard method. The diameter was measured twice, the second measurement being perpendicular to the first and the means determined.

2.7. REPORT

The results were reported as positive or negative. A complete inhibition of bacterial growth with a zone of inhibition of at least 2 mm radius on the surface of the medium surrounding both pieces of meat, the sample was regarded as positive. Whereas, an inhibition zone of at least 1 to less than 2 mm radius was regarded as doubtful.

3. ANALYTICAL METHOD VALIDATION

Comparison was made between the routinely used UK 6–plate test method [3–5] at the research institute and the method under study including validation.

3.1. PROCEDURES

3.1.1. Chicken meat samples

Chicken meat samples were ground and fortified with ERY, PEN, TYL, TRM, CTC, DOX, Neomycin (NEO), TIL CIP, ENR and NOR to obtain the levels of 0.5, 1 and 2 MRL; 100 g of sample was needed per level of MRL for each antimicrobial. Samples were homogenized well and frozen. Tests were conducted as mentioned above although repeating each sample ten times on every plate before the results were interpreted.

3.1.2. Milk and serum samples

Milk and serum samples were spiked with the 12 antimicrobials under study to obtain the concentration levels equivalent to 0.5, 1 and 2 MRL. In each case 10 mL of sample was

needed per each level of MRL of each antimicrobial. Samples were homogenize well and analyzed using the protocol mentioned above. Tests were repeated ten times on each agar plate.

3.1.3. Egg samples.

The egg white was aseptically separated from the yolk and the egg yolk spiked with the 12 antimicrobials to obtain the concentrations levels equivalent to 0.5, 1 and 2 MRL; 10 mL of sample per each level of MRL of each antimicrobial was required. Samples were then homogenized well before following the above protocol with tests repeated 10 times on each agar plate.

3.1.4. Honey samples

Samples were diluted with phosphate buffer to 1:1 (w/v), spiked with the 12 antimicrobials; 10 mL of sample is needed per level of MRL of each antimicrobial. Samples were homogenized well and tests conducted according to the protocol above with tests repeated 10 times on every agar plate.

3.2. PRELIMINARY CONCLUSION

3.2.1. Chicken meat samples

The method under study could detect 0.5 MRL of CIP and ENR; 1 MRL of DOX, NOR and PEN G; and 2 MRL of ERY and TIL while the UK 6–plate test method could detect 0.5 MRL of CIP, ENR, CTC and DOX; 1 MRL of NOR; and 2 MRL of ERY and PEN G.

For Chicken meat samples in conclusion, the method studies had the same sensitivity for CIP, ENR and NOR as the UK 6–plate method, which was however more sensitive than the method under study with regard to CTC and DOX.

3.2.2. Milk samples

The method under study could detect 0.5 MRL of CIP, ENR, NOR, DOX, NEO and TIL (on PCA8 + *K. rhizophila for TIL*); 1 MRL of ERY; and 2 MRL of OTC while the UK 6–plate test method could detect 0.5 MRL of CIP, ENR, NOR. DOX and NEO; 1 MRL of TIL; and 2 MRL of ERY, CTC and OXY.

The method under study was more sensitive than the UK 6–plate method overall. However, inclusion of the plate PCA 8 or MHA 7.2 + *K. rhizophila* to the UK 6–plate test [3–5] resulted in both methods having the same sensitivity.

3.2.3. Honey sample

The method under study could detect CIP, ENR, NOR, DOX and TIL at 0.5 MRL; and ERY, NEO and OTC at 1 MRL while the UK 6 plate method could detect CIP, ENR, NOR and DOX at 0.5 MRL and CTC, OTC, NEO and TIL at 1 MRL. The UK 6 method could detect ERY and PEN G at 2 MRL.

In conclusion the UK 6–plate method was more sensitive than the method under study especially if the plate of MHA 7.2 or PCA 8 + *K. rhizophila* is included in the method.

3.2.4. Serum samples

The method under study could detect 0.5 MRL CIP, ENR, DOX, NEO and TIL; 1 MRL ERY; and 2 MRL NOR. The UK 6–plate method could detect 0.5 MRL CIP, ENR, NOR, DOX, NEO and TIL; 1 MRL CTC; and 2 MRL ERY and PEN G.

In conclusion, the UK 6 plate method had the same sensitivity for CIP, ENR, DOX, NEO and TIL though generally more sensitive than the method under study especially when the plate of MHA 7.2 or PCA 8 + *K. rhizophila* was included.

3.2.5. Egg yolk samples

The method under study could detect 0.5 MRL CIP and ENR; 1 MRL ERY; and 2 MRL DOX, NOR, PEN and TIL while the UK 6–plate method could detect NOR; and PEN in addition at 0.5 MRL; TIL at 1 MRL and 2 ERY and DOX MRL. Therefore, in conclusion, the UK 6 – plate method was more sensitive than the method under study.

4. CONCLUSION

From the validation data, the UK 6–plate method as used at VPHL had better sensitivity than the method under study. However, the results of this study contain useful information for the member countries who want to do similar studies, as follows:

(1) MeCN, MeOH, MeCN+MeOH (19:1, v/v), phosphate buffer, and acetone+H_2O (2:1, v/v) are the extraction solutions that do not have inhibition effect on the microorganisms under study.
(2) Meat, egg yolk, milk, and serum samples show no matrix effect on the test but honey sample needs to be diluted with phosphate buffer (1:1, w/v) to avoid matrix effect.
(3) When comparing the method under study with the UK 6–plate method, the overall results show that the method under study is slightly less sensitive than the former. However, including *B. subtilis* to the method under study will enhance the sensitivity of the method and vice versa, if the plate MHA pH 7.2 added with *K. rhizophila* is included to the UK 6–plate method, the sensitivity of the method can be enhanced.
(4) To make the method under study more sensitive, it is necessary to concentrate the extracted sample to concentrate the residue more. However, to do so more lab facilities/instruments as well as more analysing time will be needed. That makes the method more complicated.

ACKNOWLEDGEMENT

The investigators thank the IAEA for the support.

ANNEX I.

PREPARATION OF CULTURE MEDIA, CHEMICALS AND REAGENTS

I-1. CULTURE MEDIA

I-1.1. Tryptic soy agar (TSA)

Pancreatic digest of casein	15.0 g
Peptic digest of soya bean	5.0 g
Sodium chloride (NaCl)	5.0 g
Agar	15.0 g
Distilled H_2O	1, 000 mL

This included suspening all ingredients in H_2O and boiling to dissolve completely. If necessary, the pH would be adjusted so that after sterilization it was 7.3±0.2 at 25ºC. The media was dispensed into suitable containers and sterilized by autoclaving at 121ºC for 15 min.

I-1.2. Finley and Fields Medium

Nutrient agar	18.0 g
Glucose	5.0 g
Manganese sulphate	0.03 g
Distilled H_2O	1,000 mL

This involved suspending all ingredients in H_2O and boiling to dissolve completely. As necessary, the pH was adjusted so that after sterilization it was 7.0±0.2 at 25ºC. The media was dispensed into suitable containers and autoclaved at 121ºC for 15 min.

I-1.3. Test agar at pH 6.0

Test Agar pH 6.0 (Merck)	25.0 g
Distilled H_2O	1,000 mL

This involves suspending the test agar pH 8.0 medium in H_2O and boiling to dissolve completely. As necessary, the pH was adjusted so that after sterilization it was 6.0±0.1 at 25ºC. The media (in 100 mL containers) was then autoclaved at 121ºC for 15 min.

I-1.4. Plate count agar pH 8.0 (PCA8)

Tryptone	5.0 g
Yeast extract	2.5 g
Dextrose	1.0 g
Agar	15.0 g
Distilled H_2O	1,000 mL

This included suspending all ingredients in H_2O and boiling to dissolve completely and the pH adjusted as necessary so that after sterilization it was 8.0±0.2 at 25ºC. The medium in smaller quantities was autoclaved at 121ºC for 15 min.

I-1.5. Nutrient agar (NA)

Peptone	5.0 g
Beef extract	3.0 g
Agar	15.0 g
Distilled H_2O	1,000 mL

This included suspending all ingredients in H_2O and boiling to dissolve completely; adjusting the pH so that after sterilization it was 6.8±0.2 at 25ºC; dispensing the medium in quantities into suitable containers and autoclaving at 121ºC for 15 min.

I-1.6. 0.1% Peptone normal saline solution (Diluent, DF)

NaCl	8.5 g
Peptone	1.0 g
Distilled H_2O	1,000 mL

This included suspend ingredient in H_2O; adding the diluents to flasks, bottles, or tubes as needed and sterilizing in an autoclave at 121ºC for 15 min. The pH was adjusted to be 7.0±0.2 after sterilization.

I-1.7. Phosphate-buffer saline (PBS)

Disodium hydrogen phosphate dihydrate	8.98 g
Sodium dihydrogen phosphate	2.81 g
NaCl	8.5 g
Distilled H_2O	1,000 mL

Autoclave at 121ºC 15 min pH = 7.2±0.2

I-1.8. Antibiotic Medium No. 1 pH6 (AB6)

Peptone	6.0 g
Tryptone	4.0 g
Yeast extract	3.0 g
'Lab–Lemco' powder	1.5 g
Glucose	1.0 g
Agar No. 1	11.5 g
Distilled H_2O	1,000 mL

The materials (27 g) were suspended in 1 L of distilled H_2O and boiled to dissolve completely before autoclaving at 121ºC for 15 min. The pH was maintained at 6 after sterilization.

I-1.9. Mueller Hinton Agar pH8 (MHA8)

Beef, dehydrated infusion from	300.0 g
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled H_2O	1,000 mL

The ingredients (38 g) were added to 1 L of distilled H_2O and boiled to fully dissolve the medium completely and autoclaving at 121ºC for 15 min. The pH was maintained at 8 after sterilization.

I-1.10. Mueller Hinton Agar pH 7.2 (MHA 7.2)

Beef, dehydrated infusion from	300.0 g
Casein hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Distilled H_2O	1,000 mL

The materials (38 g) were dissolved in 1 L of distilled H_2O, boiled to dissolve the medium completely and autoclaved at 121ºC for 15 min. The pH was kept at 7.2 after sterilization.

I-1.11. Chemicals and reagents (all chemicals are analar grade unless otherwise stated)

- MeOH
- Ethanol (HPLC grade)
- Sodium chloride
- Hydrochloric acid (0.1M)
- Sodium hydroxide (0.1M)
- Sodium hydrogen phosphate ($Na_2HPO_4 \cdot 2H_2O$)
- MeCN

ANNEX II.

PROCEDURE TO PREPARE THE BACTERIAL SUSPENSION

II-1. *B.cereus* ATCC 11778

II-1.1. Recovery of *B. cereus* from beads

The procedure included:

(1) Removing one vial of beads of *B. cereus* from the freezer (≤70ºC) with vials must be kept in a cryoblock at all times.
(2) Labeling the name of the microorganism on one Nutrient agar (NA) or Tryptic soy agar (TSA) plate and three NA or TSA slopes.
(3) Using aseptic techniques to open vial and carefully removing a bead by inserting a sterile needle through the hole or by using sterile inoculating forceps.
(4) Streaking the bead immediately over the surface solid medium and incubating at 30ºC ±1ºC for 16–18 h.
(5) Removing the inoculated slopes and plates from the incubator.
(6) Visually checking the cultures using the following criteria:
    (—) Is there growth?
        () If not, re–incubate and check at regular time intervals. Check that the correct time, environment and temperature for incubation have been used.
        () If there is growth, are all the colonies of the same size and biochemical testing?
            () If not, contamination has occurred at some stage and the process should be re–examined. It may be necessary to return to the freeze–dried culture.
            () If there is growth of appropriate character and biochemical testing, the culture is accepted. The plate is discarded.

II-1.2. Preparation of *B. cereus* stock inoculums

A suspension of the bacterium was inoculate on Tryptic soya agar (TSA) plates and incubated at 30ºC±1ºC for 18 h to 24 h. From a few colonies removed from one of the plates, one or more tubes of Nutrient agar (NA) were inoculated. Incubate at 30ºC±1ºC for 18 h to 24 h. Colonies were aseptically inoculated to CRYOBANK tube to obtain a density equivalent to McFarland 3 or 4 standard followed by proper mixing. As much of the cryopreservative fluid as possible was removed with a sterile pipette and the tubes stored at ≤70ºC.

II-1.3. Preparation of *B. cereus* spore suspension

The *B. cereus* culture obtained from step 1.1 was inoculated in three successive subcultures in slant TSA to reactivate the strain. In parallel, two TSA plates for isolation and confirmation of identification by gram staining were inoculated followed by incubating at 30ºC ±1ºC for 16 h to 18 h. Culture of less than 24 h old was harvested using glass beads and 2 mL peptone salt solution and the suspension transferred to the surface of 200 mL Finley and Fields sporulation medium prepared in a Roux flask. This was incubated at 30ºC±1ºC for five days and spores harvested with glass beads and 25 mL of peptone salt solution. The material was then

centrifuged at 3,000 rpm for 15 min, the supernatant discarded and the pellet re–suspended in 25 mL of peptone salt solution. Centrifugation was repeated at 3,000 rpm for 15 min, the supernatant discarded and pellet re–suspended in 50 mL of peptone salt solution and placed in an ultrasonic wave bath for 10 min. The spore suspension was stored at +4ºC.

II-1.4. Enumeration of *B. cereus* spore suspension

Enumeration of each new spore suspension was required in order to determine the spore concentration. This involved the following:

(1) Pipetting 1 mL of the spore suspension with sterile pipette into a sterile 20×150 mm test tube containing 9 mL peptone salt solution (DF) and mixing the suspension well by shaking the tube vigorously. The result was a 10^{-1} dilution.
(2) Pipetting 1 mL of 10^{-1} dilution into a sterile 20×150 mm test tube containing 9 mL DF to obtain10^{-2} dilution.
(3) Repeating step 2 until 10^{-3} to10^{-7}dilutions.
(4) Pipetting 100 µL of 10^{-5}, 10^{-6}, and 10^{-7} dilutions onto the surface of TSA plate in duplicate for each dilution, and spreading with triangular–shaped glass rod Followed by incubating the plates at 30ºC ±1ºC for 24±2 h.
(5) Selecting the plate showing between 15–150 colonies and counting all colonies and multiplying by the dilution factor.
(6) Diluting the original suspension with peptone salt solution to obtain 2×10^{6} spores/mL suspension and storing the suspension at 4ºC–6ºC up to 5 days.

II-2. *K. Rhizophila* ATCC 9341

II-2.1. Recovery of *K. rhizophila* from beads

This procedure included

(1) Removing one vial of beads of *K. rhizophila* from the freezer (≤70ºC) keeping vials in a cryoblock at all times.
(2) Labeling the name of the microorganism on one NA or TSA plate as well as three NA or TSA slopes.
(3) Aseptically and carefully removing a bead from the vial with a sterile needle passed through the hole or by using a sterile inoculating forceps.
(4) Streaing the bead immediately over the surface solid medium and incubating at 36ºC±2ºC for 48 h.
(5) Removing the inoculated slopes and plates from the incubator.
(6) Visually checking the cultures using the following criteria:
    (i) Is there growth?
        (一) If not, re–incubate and check at regular time intervals. Check that the correct time, environment and temperature of incubation have been used.
        (二) If there is growth are all the colonies of the same size and biochemical testing?
            () If not, contamination has occurred at some stage and the process should be re–examined. It may be necessary to return to the freeze–dried culture.

() If there is growth of appropriate character and biochemical testing, the culture is accepted. The plate is discarded.

II-2.2. Preparation of *K. rhizophila* stock inoculums

A subculture of *K. rhizophila* ATCC 9341 was prepared from the stock with a loop to 2 mL of peptone salt solution. The solution was also used to inoculate two TSA plates in streaks for isolation and confirmatory identification by gram staining every time prior to preparation of stock inocula. Media were then incubated at 36ºC±2ºC for 48 h. A few colonies were removed from one of the plates and inoculated into one or more tubes containing NA or TSA followed by incubate at 36ºC±2ºC for 48 h. CRYOBANK tube were inoculated with colonies to obtain a density equivalent to McFarland 3 or 4 standard followed by thorough mixing. As much of the cryopreservative fluid as possible was removed and stored at ≤ 70ºC.

II-2.3. Preparation of *K. rhizophila* stock suspension

K. rhizophila culture was inoculated in three successive subcultures in slant TSA to reactivate the strain. In parallel, two TSA plates were inoculated in streaks to isolate and confirm identification by gram staining. This was then incubated at 36ºC±2ºC for 48 h. A suspension was prepared by flushing with 2 mL sterile peptone salt solution and transferred to the surface of 200 mL of TSA prepared into a Roux flask. This was also incubated at 36ºC±2ºC for 48 h before harvesting the culture with glass beads and 50 mL of peptone salt solution.

II-2.4. Enumeration of *K. rhizophila* suspension

Enumeration of each new stock suspension was done in order to determine the germ concentration. A working suspension (2×10^6 cfu/mL) was then prepared and stored at +4ºC to 5 days.

II-3. *E. coli* ATCC 11303

II-3.1. Recovery of *E. coli* from beads

This included the following:

(1) Removing one vial of beads of *E. coli* from the freezer (≤70ºC) keeping vials in a cryoblock at all times.
(2) Labeling the name of the microorganism on one NA or TSA plate and three NA or TSA slopes.
(3) Aseptically removing a bead.
(4) Streaking the bead immediately over the surface solid medium and incubating at 36ºC±2ºC for 18–24 h.
(5) Removing the inoculated slopes and plates from the incubator.
(6) Visually checking the cultures using the following criteria:
    Is there growth?
    (一) If not, re–incubate and check at regular time intervals. Check that the correct time, environment and temperature of incubation have been used.
    (二) If there is growth are all the colonies of the same size and biochemical testing?

() If not, contamination has occurred at some stage and the process should be re–examined. It may be necessary to return to the freeze–dried culture.
() If there is growth of appropriate character and biochemical testing, the culture is accepted. The plate is discarded.

II-3.2. Preparation of *E. coli* stock inoculums and suspension

E. coli was prepared and aubculture prepared using 2 mL of peptone salt solution. Cultures were inoculated on NA or TSA and relevant densities equivalent to McFarland 3 or 4 standard prepared in a CRYOBANK tube followed by storage at ≤70ºC. An *E. coli* suspension was prepared and enumerated with a target working suspension of 2×10^6 cfu/mL.

Using *G. stearothermophilus* ATCC 10151 and *B. megaterium* ATCC 9885, the bacteria were recovered as stock and working solutions 2×106 cfu/mL were also prepared as the other bacteria above.

ANNEX III.

AGAR PLATE PREPARATION

TABLE III-1. CONDITIONS FOR PREPARATION OF AGAR PLATES

Media	pH	Abbreviated	Microorganism (2×10^6 cell/mL)	Agar mL/plate	Incubation Temp (ºC)
1. Mueller Hinton Agar	8	MHA 8	*K.rhizophila*	5	37±1
2. Test Agar	6	CM 6	*G. stearo-thernophilus*	15	62±2
3. Plate Count Agar	8	PCA 8	*K. rhizophila*	5	37±1
4. Antibiotic Medium No. 1	6	AB 6	*B. cereus*	5	37±1
5. Mueller Hinton Agar	7.2	MHA 7.2	*B. megaterium*	5	44±1
6. Mueller Hinton Agar	7.2	MHA 7.2	*G. stearothermophilus*	15	62±2
7. Mueller Hinton Agar	8	MHA 8	*E. coli*	5	37±1

This involves

- Melting the sterile agar and bringing the temperature down to 44ºC–50ºC;
- Adding 1 mL of the microorganism (concentration of 2×10^6 cells/mL) to 100 mL of the melted agar as indicated above;
- Mixing well and pouring 5 mL or 15 mL of the mixture to 10 mm petri dish as indicated above;
- Keeping the prepared plate at 2ºC–4ºC;
- Bringing the temperature to room temperature before use;

ANNEX IV.

PROCEDURE FOR PREPARATION OF ANTIMICROBIAL STANDARD SOLUTIONS

Standard solutions containing reference antimicrobial agents were used to verify that the operation conditions are systematically respected.

IV-1. PEN G SODIUM (SIGMA, REFERENCE PENNA OR EQUIVALENT)

IV-1.1.PEN stock solution

Use a 100 mL volumetric flask. Dissolve a quantity of PEN G Sodium equivalent to 100,000 IU in distilled H_2O and adjust volume to 100 mL with the same solvent. The stock penicillin solution may be stored at +4ºC to +6ºC for up to four days.

IV-1.2.PEN working solution

Prepare immediately before use as follows. Pipette 1 mL of PEN stock solution into a test tube containing 9 mL sterile distilled H_2O and mix well. Pipette 0.1 mL of the above solution into a test tube containing 9.9 mL sterile distilled H_2O. The final concentration of the solution is 0.001 IU/µL.

IV-2. STANDARD SDM (SIGMA, REFERENCE S6256 OR EQUIVALENT)

IV-2.1.SDM stock solution

Use a 50 mL volumetric flask. Dissolve a quantity of SDM equivalent to 50 mg of active substance in 5 mL of 0.1N NaOH and adjust volume with distilled H_2O to 50 mL. The stock SDM solution may be stored at +4ºC to +6ºC for up to two weeks. The final concentration of the solution is 1 mg/mL.

IV-2.2.SDM working solution

Prepare immediately before use follows. Pipette 1 mL of SDM stock solution to a test tube or volumetric flask containing 19 mL of sterile distilled H_2O and mix well. The final active SDM concentration is 0.05 µg/µL.

IV-3. STREP (SIGMA, REFERENCE S6501 OR EQUIVALENT)

IV-3.1.STREP stock solution

Use a 50 mL volumetric flask. Dissolve a quantity of STREP equivalent to 50 mg of active substance in H_2O and adjust volume with distilled H_2O to 50 mL. The stock STREP solution may be stored at +4ºC to +6ºC for up to 1 m. The final concentration of the solution is 1 mg/mL.

IV-3.2.STREP working solution

Prepare immediately before use by pipetting 1 mL of the stock solution into a test tube or volumetric flask containing 19 mL of sterile distilled H_2O and mix well. The final streptomycin concentration is 0.05 µg/µL.

IV-4. ERY (SIGMA, REFERENCE E6376 OR EQUIVALENT)

IV-4.1.ERY stock solution

Use a 50 mL volumetric flask. Dissolve a quantity of ERY equivalent to 50 mg of active substance in 3 mL of MeOH and adjust volume with distilled H_2O to 50 mL. The stock ERY solution may be stored at +4ºC to +6ºC for up two weeks. The final concentration of the solution is 1 mg/mL.

IV-4.2.ERY working solution

Prepare immediately before use by pipetting 1 mL of ERY stock solution into a test tube or volumetric flask containing 19 mL of sterile distilled H_2O and mix well. Pipetting 1 mL of the above solution and add 19 mL of sterile distilled H_2O. The final ERY concentration is 0.0025 µg/µL. The working solution can be kept at +4ºC to +6ºC for 3–4 days.

IV-5. OTC (SIGMA, REFERENCE O5875 OR EQUIVALENT)

IV-5.1.OTC stock solution

Use a 50 mL volumetric flask. Dissolve a quantity of OTC equivalent to 50 mg of active substance in 5 mL MeOH and adjust volume with distilled H_2O to 50 mL. The stock may be stored at +4ºC to +6ºC for 1 m. The final concentration of the solution is 1 mg/mL.

IV-5.2.OTC working solution

Prepare immediately before use by pipetting 1 mL of the stock solution into a sterile test tube or volumetric flask containing 19 mL of sterile distilled water and mix well. The final active OTC concentration is 0.05 µg/µL.

IV-6. CIP (FLUKA, REFERENCE 17850 OR EQUIVALENT)

IV-6.1.CIP stock solution

Use a 50 mL volumetric flask. Dissolve a quantity of CIP equivalent to 50 mg of active substance in 5 mL of 3% acetic acid and adjust volume with distilled H_2O to 50 mL. The stock may be stored at +4ºC to +6ºC for 1 m. The final concentration of the solution is 1 mg/mL.

IV-6.2.CIP working solution

Prepare immediately before use by pipetting 300 µL of the stock solution into 20×150 mm sterile test tube containing 9.7 mL sterile distilled H_2O and mix well. Pipette 100 µL of the solution above to a sterile test tube containing 9.9 mL distilled H_2O. The final CIP concentration is 0.0003 µg/µL.

IV-7. TMP (SIGMA, REFERENCE T4881 OR EQUIVALENT)

IV-7.1.TMP stock solution

Use a 10 mL volumetric flask. Ten milligrams active substance of TMP is dissolved in 10 mL ethanol under shaking at 50ºC±1ºC. Store this solution in cool and dark place, so will keep for 7 m. The final concentration of the solution is 1 mg/mL.

IV-7.2.TMP working solution

Add 0.1 mL of TMP stock solution to 1.9 mL sterile distilled H_2O. The final concentration of TMP is 50 µg/mL. Keep this solution under cold storage +4ºC to +6ºC within 14 days.

TABLE IV-1. PREPARATION OF ANTIMICROBIAL SOLUTIONS

	Chicken meat	**Pork**	**Milk**	**Serum**	**Eggs**	**Honey**
1. ERY	100[2]	200[1]	40[1]	40*	50[1]	50*
2. PEN	50[2]	50[2]	50*	50*	150[1]	50*
3. TYL	100[2]	100[2]	50[1]	50*	200[1]	50*
4. TMP	50[1]	50[1]	50[1]	50*	50*	10*
5. CTC	100[1]	100[1]	100[1]	100*	200[1]	100*
6. DOX	100[1]	100[1]	100[2]	100*	100*	10*
7. OTC	100[1]	100[1]	100[1]	100*	200[1]	100*
8. NEO	500[1]	500[1]	1500[1]	1500*	500[1]	500*
9. TIL	75[1]	50[1]	50[1]	50*	50*	50*
10. CIP	100[1]	100[1]	100[1]	100*	50*	10*
11. ENR	100[1]	100[1]	100[1]	100*	50*	10*
12. NOR	100*	100*	100*	100*	100*	10*

Note: MRLs used in this study follow set by EU CODEX either MRLs. It the MRLs set by EU and CODEX are different. The lower levels are selected.

* = No MRLs set by EU / CODEX

MRLs for milk were followed except neomycin

[1] = EU [2]

[2] = CODEX [1]

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DEVELOPMENT OF IMMUNOASSAYS FOR SELECTED ANTIBIOTICS FOR THE DETECTION AND MONITORING OF THE DRUG RESIDUES IN LIVESTOCK AND LIVESTOCK PRODUCTS

MURILLA, G., KARANJA, W., ARUSEI, J., MDACHI, R.
KARI–Trypanosomiasis Research Centre, Kikuyu, Kenya

Abstract

A direct competition Enzyme Linked Immunosorbent Assay (ELISA) test has been developed and validated for monitoring tetracycline (TC) residues in edible animal products with a limit of detection (LOD) of 10 ng/mL plasma. The antibody produced in chicken egg gave high titres enabling development and optimization of the assay. The antibody and drug conjugate titres obtained during assay optimization were 1 in 51,200 and 1 in 12,800, respectively.

High levels of antibodies were obtained in rabbits and chicken eggs following immunization. This ensured high dilutions in the assays, and therefore low reagent volumes. Antibodies produced increased and matured with subsequent booster injections with the rabbit requiring more booster injections than chicken. At 34 samples per microtitre plate (in duplicate), 1 mL of antibody would be sufficient for analysis of approximately 100,000 samples. The semi–log plot of optical densities (ODs) of standard solutions vs concentration gave a linear calibration curve. Although the assay results were reproducible, high background reactions were observed.

1. INTRODUCTION

Presence of veterinary drug residues in edible animal products remains the greatest of challenge to regional trade and consumer health safety. Widespread drug resistance has also been reported. Studies undertaken in Kenya have demonstrated the presence tetracycline residues in the various edible tissues exceeding maximum recommended levels (JECFA Evaluation [1–4]. Although regulations exist on withdrawal periods following treatment and before slaughter, these are neither followed nor enforced. In order to effectively monitor drug levels in animal products, accurate and easy to apply methods are required for the detection and monitoring of circulating drug levels in cattle at slaughterhouses to determine when an animal should be slaughtered. The Trypanosomiasis Research Centre has been working in collaboration with Kenya Meat Commission of Kenya for over 10 years, monitoring veterinary drug residue levels in meat products collected from various parts of the country. This was to help establish the extent and severity of the problem for government intervention. The centre has successfully developed in house analytical procedures for trypanocidal drugs which are currently in use. This paper reports an ELISA method for the detection of tetracycline and oxytetracycline in animal products. This assay has an LOD of 10 ng/mL.

Tetracylines are a group of antibiotics with broad spectrum of activity. The most commonly used tetracyclines in veterinary medicine, due to their low toxicity profile and low cost, are TC, oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DOX). Tetracylines inhibit protein synthesis. In food producing animals, the antibiotics are often used as feed additives. Whereas these veterinary products contain information on withdrawal periods, this is often ignored by farmers, resulting in violative Maximum Residue Levels (MRLs) in products such as milk and meat [5–7]. The MRLs set by the Codex Alimentarius Commission for TC, OTC and CTC in cattle muscle, liver, kidney and milk are 200 μg/kg, 600 μg/kg, 1,200 μg/kg and 100 μg/kg, respectively [5–7].

In Kenya, high levels of TC residues have been detected in liver kidney and muscle samples collected from slaughterhouses in Nairobi [8]. Cattle that are slaughtered and sold in and around Nairobi are sourced from various parts of the country. Two hundred and fifty beef samples collected from five slaughterhouses in and around the city of Nairobi were analysed

for TC residues using the High Performance Liquid Chromatography (HPLC). Of the 250 samples analysed for tetracycline residues, 114 (45.6%) had tested positive. The 114 samples included 60 (24%) liver, 35 (14%) kidney and 19 (7.6%) muscle. The detected residues were OTCs in 110 (44%) samples and CTCs in four (1.6%) samples. The mean residue levels of the detected tetracyclines were higher than the MRLs in edible tissues [5–7]. Previous studies have also demonstrated that high drug residues persist for long periods of time in edible animal products following treatment [8–10]. Using Charm–AIM, Aboge *et al.*, [11] reported 9.4% and 5.7% milk samples from Kenyan consumer households and market agents with microbial agents above EU MRLs, respectively.

In view of the above, it is clear that veterinary drug residues are a major problem perhaps not only in sub–Sahara Africa but also other countries, resulting from non–adherence to recommended withdrawal times after drug administration. These require regular monitoring in order to protect the consumer. Accurate, easy to apply and cost–effective screening methods are therefore required for routine monitoring of animals in slaughterhouses and also animal products on the market as way of controlling residues. Several methods have been reported [12–14] which are either too long, with several extraction steps, or expensive for routine use, covering large areas and high numbers of samples, hence the aim of this study of developing and validating a multi–residue ELISA test for monitoring tetracycline residues in edible animal products.

2. MATERIALS AND METHODS

All chemicals and reagents used were of analytical grade. Water was de–ionized and triple distilled.

2.1. ANTIBODY AND ANTIGEN PRODUCTION AND PURIFICATION

2.1.1. Selection of animals for antibody production

Rabbits and chicken were selected for the production of polyclonal antibodies against tetracyclines (TC, CTC and OTC). This selection was made on the basis that the antibodies produced would allow development of tests applicable to a wide range of food producing animals. Therefore, the use of rabbit and egg–laying chicken was found appropriate. Production of antibodies in chicken has a number of advantages. First, the maternal antibodies are transferred to the egg yolk where they are collected non–invasively. The antibodies have a half–life of six months (m) at room temperature or 1 m at 37°C. The quantity of immunogen required for an efficient immune –response is much lower than for the rabbits [15, 16].

This work was undertaken in adherence to experimental guidelines and procedures approved by the Institutional Animal Care and Use Committee (IACUC) at the Kenya Agricultural Research Institute's Trypanosomiasis Research Centre (KARI–TRC). These IACUC regulations conformed to guidelines provided by the Kenya Veterinary Association.

2.1.1.1.Preparation of TC/CTC–KLH immunogen

Keyhole limpet hemocyanin (KLH) was identified as the appropriate carrier protein for preparation of the immunogen. Coupling of KLH to tetracyclines was enabled by introduction of a carboxylic group through diazotization while N–hydroxy–succinamide ester (NHS) was used as cross–linker. Antibody purification was carried out following the procedure described below. In brief, 69 mg TC and 53 mg of CTC were dissolved in 2 mL 2M NaOH in a bijou bottle (Solution A). On an ice–bath a solution of $NaNO_2$ (22 mg in 1 mL H_2O) was added to a

solution of 4–aminobenzoic acid (ABA, 37 mg in 1 mL 2M HCl), with constant stirring. The mixture was left standing for 30 min (Solution B). Solution B was added to Solution A on an ice bath and the reaction left to continue for 2 h after which the pH was adjusted to 8.7 using dry Na_2CO_3. One mL 6M HCl was then added, and the resulting precipitate filtered, recovered and stored in the dark at room temperature. Then 68 mg of the recovered precipitate were dissolved in dimethylformamide (Solution C).

Solution C was added, slowly, gently and with stirring to another solution of N–hydroxysuccinamide (NHS; 36 mg in 0.2 mL DMSO), followed by a solution of KLH (20 mg in 2 mL of de–ionized triple distilled H_2O). The combination was left mixing for 4 h at room temperature after which it was dialysed overnight with three changes of 0.9% saline. The conjugate was aliquoted into 2 mL vials in volumes of 0.5 mL and stored at -20°C until required for use.

2.1.1.2.Preparation of Tetracycline-EDC conjugate

The enzyme conjugates for tetracyclines were prepared via the active ester method using N–(3–Dimethylaminopropyl)–N–ethylcarbodiimide hydrochloride (EDC) and glutaraldehyde. A tetracycline horseradish peroxidase conjugate was also prepared by coupling the two with either para–benzoquinone (PBQ), sodium chloroacetate or 4–aminobenzoic acid (4–ABA, Sigma, Aldrich, UK). The reagents were mixed in the proportions of 1:1:1.

A 6 cm dialysis tubing Viking size 2.18/32" (Medicell International, London) was boiled in double distilled H_2O for 15 min. The tube was filled with conjugate and dialyzed against 0.9% saline for 7 h with two changes at equal intervals. Further purification was achieved with addition of activated charcoal followed by centrifugation at 10,000 rpm for 30 min. The supernatant was separated and an equal volume of glycerol (Sigma, UK) added and stored at -20ºC.

2.1.1.3.Immunization of rabbits and monitoring of antibody production

Groups of two rabbits each were immunized with either, a mixture of TC and OTC immunogen or a mixture of TC, OTC and CTC immunogen. The priming dose was given in Freund's complete adjuvant while booster doses were given every 21 days in incomplete Freund's adjuvant with test bleeds one every two weeks for 5 m.

2.1.1.4.Immunization of egg–laying chicken and monitoring of antibody production

Pre–immunization blood was collected a day before injection, sera prepared and stored at -20°C until required. Primary injection containing 0.25 mg protein in Complete Freund's Adjuvant (CFA) was administered subcutaneously at 4 different sites. This was followed by two booster immunizations of 0.10 mg protein in Incomplete Freund's Adjuvant (IFA) at 14 and 42 days after the primary dose. Blood was collected from chicken on days 21, 48 and 56, serum prepared and stored. Eggs were collected for 3 weeks between days 21 and 42 and for one week each from day 48 and day 56 after primary dose.

2.1.1.5.Purification of antibody

Rabbit anti–sera

The antisera produced in the two groups of rabbits were used without further purification. Following separation the anti–sera was aliquoted (0.5 mL volumes) into 2 mL tubes and stored at -20°C until required for use.

IgY for egg yolk

The purification of the IgY from egg yolk was carried out as described elsewhere [17] as detailed in Annex I.

2.2. ASSAY DEVELOPMENT

2.2.1. Determination of antibody/antigen titres

Determination of antibody and antigen titres was done in checkerboard titration using a 96 – well micro–titre plate (Immulon 4, Dynatech, UK). The antibody/antigen combination that gave an optical density of approximately 1 was selected for subsequent optimizations. The micro–titre plate was coated in doubling dilution across the plate, incubated overnight at +4°C, frozen at -20°C and thawed only when required for use. Once thawed and washed in PBST, the drug conjugate was added in doubling dilution down the microtitre plate. The plates were incubated for 30 min, washed, and colour developed using TMB/hydrogen peroxide substrate/chromogen. The reaction was stopped using phosphoric acid and the ODs of the yellow solution determined in an ELISA plate reader at the wavelength of 450 nm.

2.2.2. Negative control sera

Ten ml blood was collected from cattle in vacutainer tubes, plasma separated and stored at -20°C until required. Each individual sample was tested in the ELISA before pooling for use as negative control plasma. This control plasma was used in the preparation of standards.

2.2.3. Whole plate CVs

As part of assay optimization and in order to assess possible variations of results due to the solid phase (microtitre plate), the whole plate CVs were determined. This was carried out by analysing negative control sera in replicate and calculating the CVs.

2.2.4. Spiked TC standard solutions

Standards of TC, OTC and CTC were separately dissolved in H_2O at the highest concentration of 500 ng/mL. A series of dilutions (3–fold) were prepared in control plasma/serum, aliquoted, and stored at -20ºC. Eight dilutions that gave a linear calibration curve were used for subsequent analyses. The results obtained were used to construct a calibration curve from which concentrations of test samples were extrapolated.

2.2.5. Cross reactivity

Cross reactivity was investigated using other antibiotics commonly used in the treatment of livestock diseases in Kenya. Cross reactivity was expressed as the dose of drug required to reduce by 50% the max OD obtained in the absence of the drug.

2.2.6. Assay LOD

This was carried out by assaying zero dose plasma/serum in duplicate (n=10). The mean ODs, the standard deviation were determined. B_0 is the OD obtained from the calibration curve (extrapolated) at zero dose and B_0 = ODs of individual animal plasma/serum. A concentration corresponding to the Mean minus one SD, read from the calibration curve was taken as the least detectable dose.

2.2.7. Assay validation

Oxytetracycline (Adamycin®) was administered to a group of four sheep; 10 mL blood collected in heparinized vacutainer tubes at pre–determined intervals, the plasma prepared and stored at -20°C. These samples were analysed as described above, ODs obtained and concentrations determined from the calibration curve.

3. RESULTS AND DISCUSSION

Checkerboard titrations carried out to determine optimal dilutions for antibody and conjugate for use in assay development showed that the antibody produced in the rabbit matured with time following booster immunizations. However, low titres were observed with the immunogen prepared from a cocktail of TC, OTC and CTC.

3.1. EVALUATION OF ANTI–TC POLYCLONAL ANTIBODIES PRODUCED IN RABBITS

Figures 1 (a) and (b) show the profiles of antibodies produced in rabbit 7 against a cocktail of TC, OTC and CTC. The ODs obtained were very low.

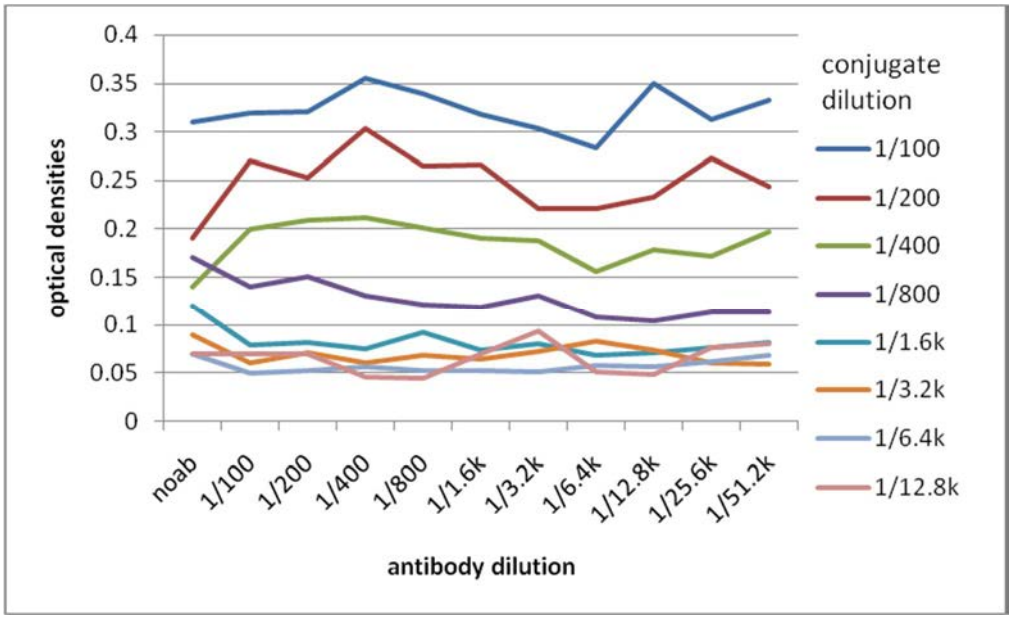

FIG. 1 (a). Day 35 after the last booster immunization.

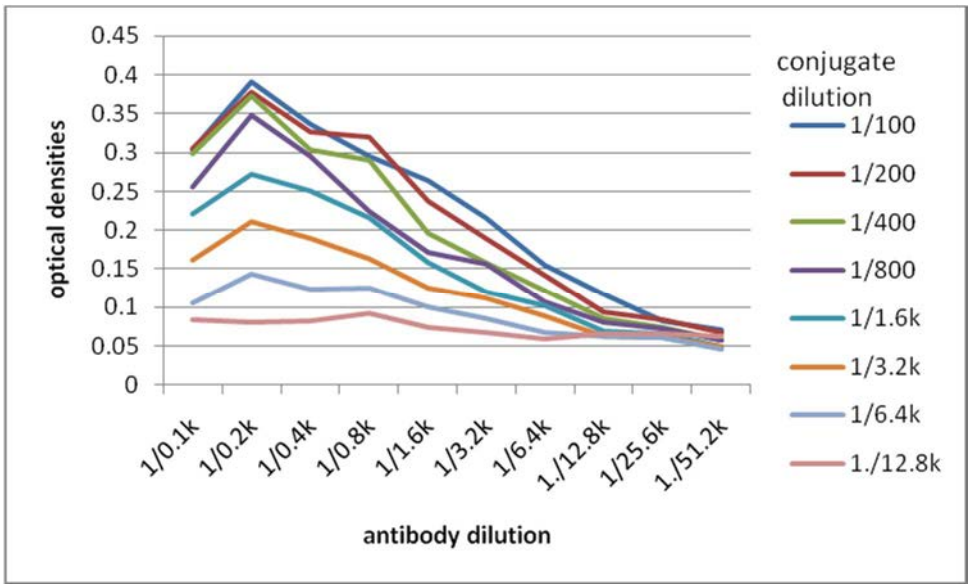

FIG. 1 (b). Day 49: Shows maturation of antibody with time after the last booster immunization dose.

Figure 2 shows checkerboard titration of rabbit 6 antisera obtained from immunization with OTC/TC/CTC immunogen. The OTC–HRP conjugate was prepared via sodium Chloro–acetate.

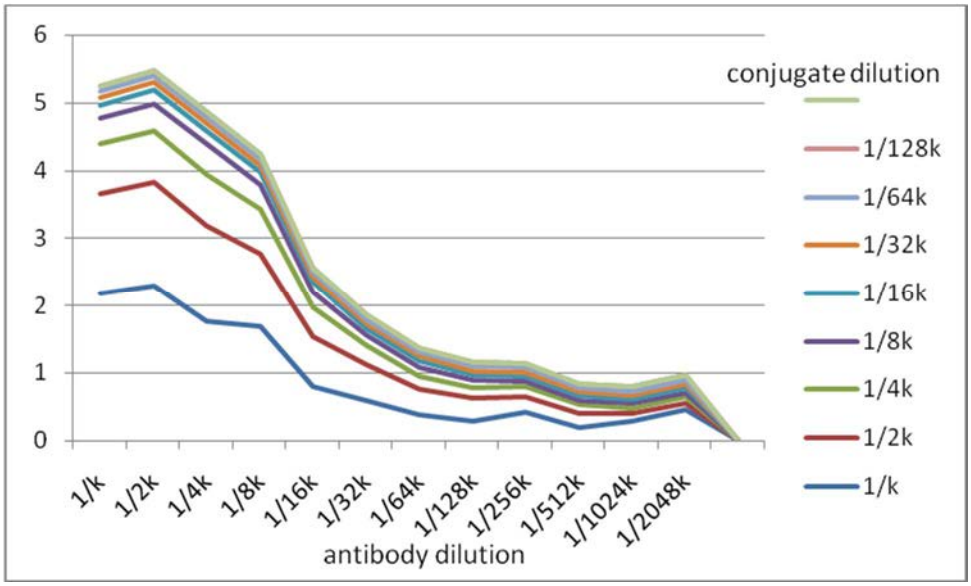

FIG. 2. Checkerboard titration of rabbit 6 antisera obtained from immunization with OTC/TC /CTCimmunogen.

Figure 3 shows checkerboard titration of rabbit 4 antisera obtained from immunization with OTC/TC immunogen. OTC–HRP conjugate was prepared via sodium Chloro–acetate and EDC.

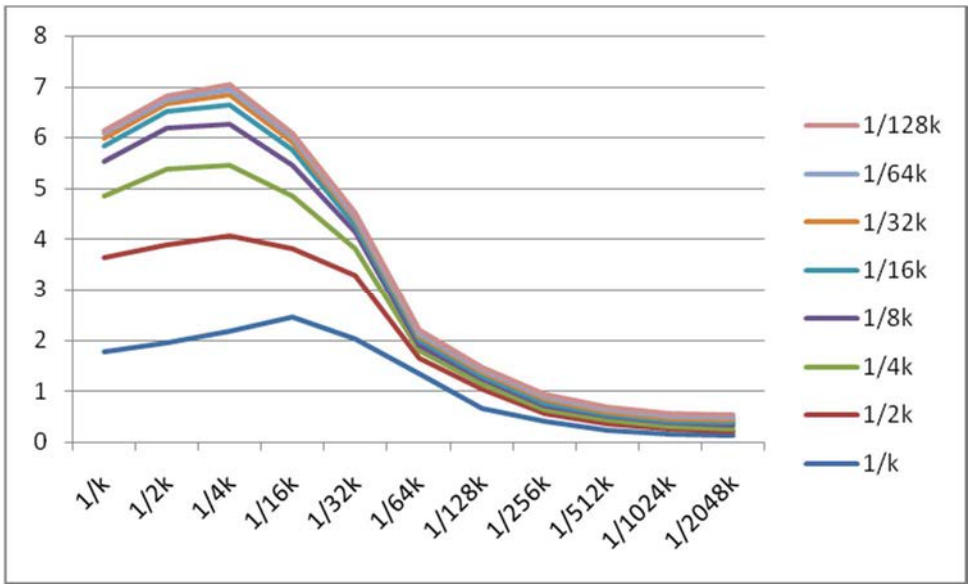

FIG. 3. Checkerboard titration of rabbit 4 antisera obtained from immunization with OTC/TC immunogen.

3.2. EVALUATION OF ANTI–TETRACYCLINE POLYCLONAL ANTIBODIES PRODUCED IN CHICKEN EGGS

Figure 4 shows checkerboard titration of chicken egg IgY obtained on day 40 following the last booster immunization dose with OTC/TC immunogen. OTC–HRP conjugate was prepared via sodium chloro–acetate and EDC.

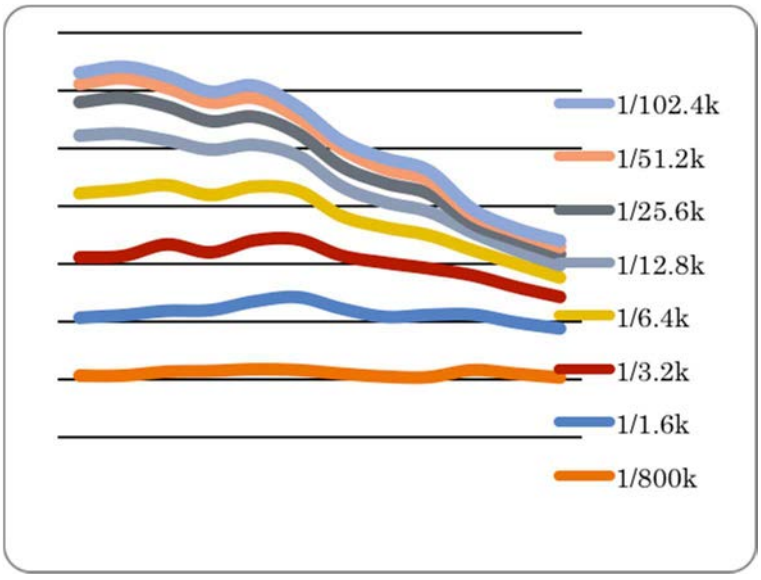

FIG. 4. Checkerboard titration of chicken egg IgY on day 40 after last booster immunization dose with OTC/TC immunogen.

Very high antibody titres were obtained from chicken egg following immunization with the TC–KLH immunogen. The selected optimal dilutions from the checkerboard titration were 1/51,200 for antibody and 1/12,800 for TC–HRP conjugate. However, high background reactions were observed indicating non-specific binding of conjugate to antibody.

3.2.1. Calibration Curve

Figure 5 shows a calibration curve obtained following analysis of spiked plasma samples using optimal dilutions from the checkerboard titration were 1/51,200 for antibody and 1/12,800 for tetracycline–HRP conjugate in a competitive ELISA.

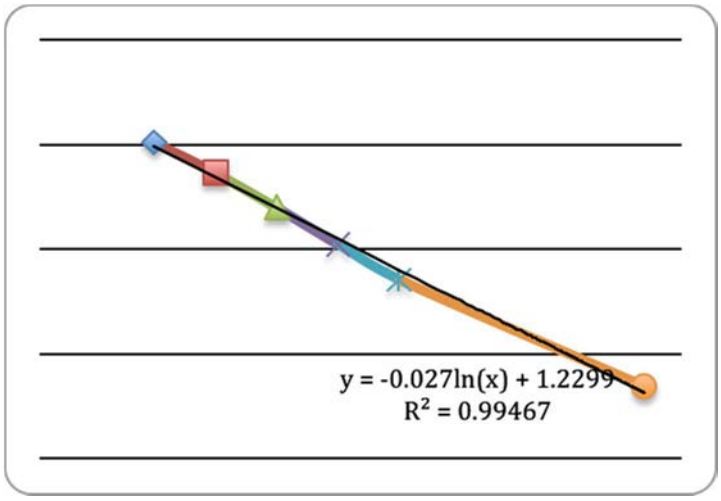

FIG. 5. Calibration curve of tetracycline in plasma.

Several calibration curves were prepared in plasma, serum and PBS. High titres were obtained when either plasma or serum was used to prepare the standard solutions. However, high background was observed which indicated non–specific binding.

3.2.2. Whole plate CVs

CVs of all the replicates were below 12% demonstrating the integrity of the solid phase (Immunlon 4, Dynatek, UK) used in the development of the assay.

3.2.3. Cross–reactivity

The most commonly used TCs in Kenya are OTC and CTC (KARI–TRC, unpublished data). Cross reactivity was demonstrated amongst the tetracyclines.

3.2.4. Assay validation

High drug concentrations were obtained following administration Adamycin® to sheep at 100 mg/kg bodyweight. However, marked variations in concentrations were observed between individual animals over time. Figure 6 shows the time vs concentration profiles obtained following administration of Adamycin® to sheep at 100 mg/kg bodyweight.

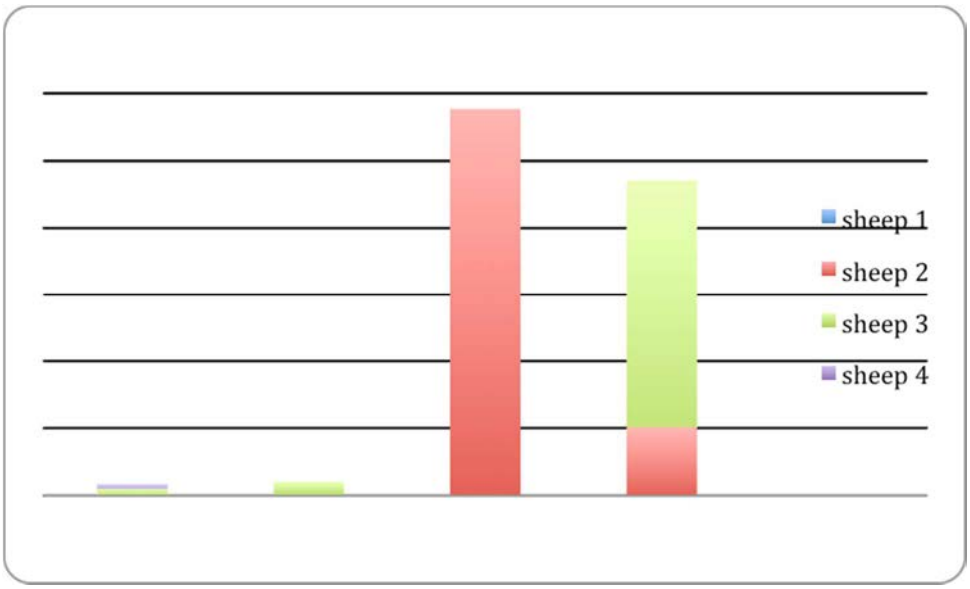

FIG. 6. Time vs concentration profiles following administration of Adamycin® to sheep at 100 mg/kg bodyweight.

The objective of this study was to develop and validate an ELISA test for monitoring tetracycline residues in edible animal products. A direct competition assay has been developed and validated with an LOD of 10 ng/mL plasma. The antibody produced in chicken egg gave high titres enabling the assay to be developed and optimized. The antibody and drug conjugate titres obtained during assay optimization were 1 in 51,200 and 1 in 12,800, respectively.

The high antibodies obtained in rabbits and chicken eggs by the immunization procedures described allowed the use of high dilutions in the assays, hence low volumes of reagents. It was shown that the antibodies produced increased and matured with subsequent booster injections. However, the rabbit required more booster injections than chicken. At 34 samples per microtitre plate (in duplicate), one ml of antibody would be sufficient for analysis of approximately 100,000 samples. The semi–log plot of ODs of standard solutions vs concentration gave a linear calibration curve. The LOD of 10 ng/mL was determined from the calibration curve using ODs of control plasma (zero dose) obtained from untreated cattle. Although the assay results were reproducible, high background reactions were observed.

Presence of veterinary drug residues in edible animal products, are of great public health and consumer health concern [4]. The MRL set for the TCs by European Union in cattle muscle is 100 µg/kg. Regulation 37/2,010 [7] also lists pharmacologically active substances and their MRL in foodstuffs of animal origin. The MRLs of TC and CTC set by FAO/WHO are: 100 mg/kg for muscle (cattle, pigs, poultry), 300 mg/kg for liver (cattle, pigs, sheep, poultry), 600 mg/kg for kidney (cattle, pigs, sheep, poultry), and 200 mg/kg for eggs (poultry). These values were updated in 2012 at the 35th session of the Codex Alimentarius Commission [18]. It is in view of the above that many analytical methods have been developed to monitor the levels of veterinary drugs in animal products.

ELISA Kits have been developed for the detection and monitoring of tetracycline in milk, honey and tissue samples with detection limits varying from 5 µg/kg in honey to 80 µg/kg in tissue [19]. Indirect ELISA kits have also been developed and are on the market. The major challenge on the access and use of these kits is the cost, especially for routine monitoring.

Countries of sub–Sahara Africa that are in the process of establishing frameworks for routine monitoring of food products of animal origin for veterinary drug residues, may find this expensive and not sustainable in the long term.

4. CONCLUSION

Commercially available kits have been developed capable of detecting levels of 5 µg/kg and below; levels that are much below the recommended MRLs. These methods may be useful in determining the extent of exposure of animals to veterinary drugs, and drug usage in any given locality. This will be important in determining areas where drug use is rampant and withdrawal periods not observed and also how these low levels contribute to the development of resistance to antibiotics in both humans and livestock.

The major challenge with the methods developed and reported in this paper is high background reactions unrelated to the analyte of interest. More validation work is therefore being undertaken to identify the sources of the non–specific binding observed.

ACKNOWLEDGEMENT

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ANNEX I.

IgY TECHNOLOGY: EXTRACTION OF CHICKEN ANTIBODIES FROM EGG YOLK BY POLYETHYLENE GLYCOL (PEG) PRECIPITATION [17]

I-1. PROTOCOL – IGY– EXTRACTION BY MEANS OF PEG–PRECIPITATION

(1) The eggshell is carefully cracked and the yolk is transferred to a "yolk spoon" in order to remove as much egg white as possible.
(2) The yolk is transferred to a filter paper and rolled to remove remaining egg white, and then the yolk skin is cut with a lancet or a similar instrument (pipette tip). The yolk is poured into a 50 mL tube and the egg volume is registered (v1).
(3) Twice the egg yolk volume of pbs is mixed with the yolk (∑v1+v2), thereafter 3.5% peg 6,000 (in gram, pulverized) of the total volume is added and vortexed, followed by 10 min rolling on a rolling mixer. That step of the extraction procedure separates the suspension in two phases. One phase consists of "yolk solids and fatty substances" [20] and a watery phase containing IgY and other proteins.
(4) The tubes are centrifuged at 4°C for 20 min (10,000 rpm according to 13,000g, Heraeus Multifuge 3sr+, fixed angle rotor). The supernatant (v3) is poured through a folded filter and transferred to a new tube.
(5) % PEG 6000 in gram (calculated according to the new volume) are added to the tube, vortexed and rolled on a rolling mixer as in step 3.
(6) Repeat step 4 with the difference that the supernatant is discarded.
(7) The pellet is carefully dissolved in 1 mL pbs by means of a glass stick and the vortexer. PBS is added to a final volume of 10 mL (v4). The solution is mixed with 12% peg 6,000 (w/v, 1.2 g) and treated as in step 3 (vortex, rolling mixer).
(8) Repeat step 6 and dissolve the pellet carefully in 800 µL PBS (glass stick and vortex). Wait for the air bubbles to disappear and then transfer (pipette) the extract to a dialysis capsule. Rinse the tube with 400 µL PBS and add the volume to the dialysis device (v5). For preparation of dialysis devices and membranes see appendix 2 below.
(9) The extract is dialysed over night in 0.1% saline (1,600 mL) and gently stirred by means of a magnetic stirrer. The next morning, the saline is replaced by PBS and dialysed for another three hours.
(10) Thereafter the IgY–extract is pulled from the dialysis capsule by a pipette and transferred to 2 mL tubes. The final volume is around 2 mL (v6).
(11) The protein content (mg/mL) of the samples is measured photometrically at 280 nm (1:50 diluted with PBS) and calculated according to the lambert–beer law with an extinction coefficient of 1.33 for IgY. Purity and recovery are around 80%.
(12) It is advisable to store the samples in aliquots at -20°C (do not freeze the samples at -70°C).
(13) the quality of the final preparations is analysed by simple SDS–PAGE as described elsewhere [21].

Before use the dialysis bag must be prepared in the following way according to the recommendations of the manufacturer:

(1) Dialysis bags are cut in pieces of 30 cm and given in a 2,000 mL glass beaker.
(2) 1750 mL of a 5 mm EDTA–solution are added.

(3) A glass funnel is placed above the bags to ensure that the bags are covered by the EDTA solution. The solution is heated and boiled for 5 min (hot plate). The solution is decanted and the bags are washed three times with distilled H_2O.
(4) Once again 1,750 mL EDTA–solution are added, boiled for 5 min and washed three times with distilled H_2O as above.
(5) Finally the dialysis bags are boiled for 10 min in distilled water and stored at 4°C. Take out the dialysis bags by means of sterilized tweezers.

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CHARACTERIZATION BY ENZYME LINKED IMMUNOSORBENT ASSAY AND BIOSENSOR TECHNOLOGIES OF ANTIBODIES TO CHLORAMPHENICOL PRODUCED IN CAMEL, DONKEY AND GOAT

FODEY, T[*]., MURILLA, G[**]., CANNAVAN, A[***]., ELLIOTT, C[+]
[*] Agri-Food and Biosciences Institute, Veterinary Sciences Division, Belfast, UK.
[**] Kenya Agricultural Research Institute, Residue Analysis Section, Kikuyu, Kenya
[***] International Atomic Energy Agency
[+] Agri-Food and Land Use, Queens University Belfast, UK

Abstract

Six antibodies against Chloramphenicol (CAP) were produced in camels, donkeys and goats and evaluated for sensitivity using Enzyme Linked Immunosorbent Assay (ELISA) and biosensor assay. The effect of matrix and other drugs was also studied. The Biosensor method showed limited cross reactivity than ELISA although with a corresponding reduction in sensitivity.

1. INTRODUCTION

Chloramphenicol (CAP) is prohibited for use in food producing animals such as in the European Community [1, 2]. This requires methods meeting minimum required performance limit (MRPL) of 0.3 μg/kg [3] given the sensitivity of the issue there is no Codex MRL [4] and the compound has been banned from use in food producing animals in many other countries and regions. It is included in the list of compounds which should not be detected in food in Japan's 'Positive List' of Maximum Residue Limits (MRL) for Agricultural and Veterinary Chemicals which took effect in May 2006. Related drugs such as Thiamphenicol have been evaluated for use [5].

A typical testing scheme for the presence of the antibiotics employs a rapid high throughput screening assay followed by confirmatory analysis using an analytical chemical method. Various methods have been reviewed including their strengths and weekneses [6–15]. Thus two rapid techniques ELISA and biosensor assay technologies to detect CAP residues using polyclonal antibodies raised against CAP in camels, donkeys and goats have been studied [16].

2. MATERIALS AND METHODS

2.1. REAGENTS AND CHEMICALS

The following were used: Isobutylchloroformate , 4–methyl morpholine CAP (C–0378), CAP succinate (C–3787), CAP base, thiamphenicol (THI), florfenicol (FFC), human serum albumin (HSA), Freunds adjuvant complete and incomplete and florfenicol amine [16]. Others were Strata X solid–phase extraction (SPE) cartridges, PD–10 Gel filtration columns horseradish peroxidase (HRP) as well as Optical Surface Plasmon Resonance (SPR) Biosensor system (BIACORE® Q), sensor chip, N–hydroxysuccinimide (NHS) coupling solution and 1–ethyl–3–(3–dimethylaminopropyl) carbodiimide hydrochloride (EDC) coupling solution [16].

2.2. EXPERIMENTAL SAMPLES

Bovine milk and porcine kidney samples that were known to be free from veterinary drugs were used to determine the matrix effect on the ELISA and biosensor assays.

2.3. PREPARATION OF CAP IMMUNOGEN, CAP ENZYME LABEL AND BIOSENSOR CHIP, ENZYME AND BIOSENSOR CHIP LABEL AS WELL AS IMMUNOGENIC EMULSION USING FREUNDS ADJUVANT

The immunogen was prepared from human serum albumin (in sodium acetate solution) and activated CAP succinate dissolved in dimethylformamide (DMF), N–methyl morpholine, Isobutylchloroformate. A CAP enzyme label was prepared by coupling CAP succinate to horseradish peroxidase; preparation of a biosensor chip is also detailed [16] The THI enzyme label was prepared through interaction between horseradish peroxidase dissolved in 1 mL of PBS and the drug. Other reagents involved included disuccinimidyl carbonate and dimethylaminopyridine dimethyl sulphoxide and sodium acetate solution. The THI biosensor chip and immunogen emulsion including use were also prepared [16].

2.4. IMMUNISATION OF ANIMALS AND ANTIBODY SENSITIVITY; ANTIBODY SENSITIVITY USING A BIOSENSOR; MATRIX EFFECTS AND SENSITIVTY ASSESSMENT.

The CAP immunogens were administered subcutaneously in the camels, donkeys and goats in decreasing concentrations and a competitive ELISA conducted to measure the sensitivity of the antibodies and IC_{50} [16]. The CAP standards diluted in HBS buffer injected over the sensor chip and points were recorded before and after each injection [16]. The effect of bovine milk and kidney samples on method sensitivity was also evaluated including use of Strata X SPE cartridges Samples were homogenized/agitated, extracted in relevant solvent and defatted prior to analysis [16].The cross recativity with THI, FFC and florfenicol amine (FFA) was also evaluated.

3. RESULTS AND DISCUSSION

3.1. ANTIBODY SENSITIVITY, SPECIFICTY AND MATRIX EFECTS

All of the animals immunised with the CAP–HSA conjugate produced responses that were detected by both the ELISA and biosensor methods [16]. The IC_{50} values in buffer ranged from 0.31 ng/mL to 5.47 ng/mL of CAP when using the ELISA and 0.72 ng/mL to 1.66 ng/mL with the biosensor. Use of milk improved ELISA method sensitivity unlike the kidney samples [16]. No significant cross reactivity was observed between CAP and its analogues THI, FFC and FFA whenusing CAP–HRP except with the antibody G2, which exhibited crosss reactivity of 1.2% and 18% for THI and FFC, respectively. Use of THI–HRP resulted in increased cross–reactivity [16]. The trend was almost the same using the biosensor method

The use of larger animals [16] had several advantages over techniques [7, 8, 10–13, 15], such as more serum without salvaging the animals, and the antibodies displayed excellent sensitivities when assessed by both ELISA and biosensor [16]. The antibody C1 was been successfully applied in a competitive ELISA for the determination of CAP in sheep sera [17, 18].

Appreciable binding occur to THI and FFC was observed only when CAP was not incorporated in the enzyme label or the chip surface [16]. The CAP based chip surface prepared was comparable to work done elsewhere [15] involving biosensor assay for CAP, THI, FFC and FFA but in a rabbit. Heterologous assays showed more cross reactivity than the homologous [16].

4. CONCLUSION

Antibodies very sensitive to CAP following analysis with ELISA and Biosensor were produced in camels, donkeys and goats. Matrix effects were recorded with milk showing no effect on sensitivity compared to kidney samples. Homologous and heterologous assays showed different results.

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DEVELOPMENT OF AN ENZYME–LINKED IMMUNOSORBENT ASSAY FOR CHLORAMPHENICOL IN SERUM

WESONGAH, J.O[*]., MURILLA, G.A[*]., GUANTAI, A.N[**]., ELLIOTT, C.T[***]., FODEY, T[+], CANNAVAN, A[++]
[*] Kenya Agricultural Research Institute, Residue Analysis Section, Kikuyu, Kenya
[**] The University of Nairobi, College of Health Sciences, Faculty of Pharmacy, Department of Pharmacology and Pharmacognosy, Nairobi, Kenya
[***] Agri–Food and Land Use, Queens University Belfast, Belfast, U.K
[+] Agri–Food and Biosciences Institute, Veterinary Sciences Division, Belfast, UK
[++] International Atomic Energy Agency

Abstract

A competitive enzyme linked immuno–sorbent assay (ELISA) with a detection limit (LOD) of 0.1 ng/mL was developed and optimized for the detection of Chloramphenicol (CAP) in sheep serum involving CAP–horseradish peroxidase conjugate and camel antibodies as well as tetramethylbenzidine–hydrogen peroxide as a chromogen–substrate system. The assay had a high specificity for CAP and could thus be used to screen CAP in animal products with limited cross–reactivity with a number of related drugs.

1. INTRODUCTION

Various methods can be used for analysis of prohibited drug CAP, namely [1–5] although appropriate commercial conjugates and the antibodies are either not readily accessible or expensive and reagents have a short shelf–life. There was therefore need to develop (in–house) an affordable ELISA kit to monitor livestock products for local consumption and export [6].

2. MATERIAL AND METHODS

2.1. EXPERIMENTAL ANIMALS AND SAMPLES

As reported elsewhere, [6] two camels, goats and donkeys each were included in the study, fed ad lib and treated again ectoparasites. Sheep were also included. The preparation of the immunogen and production of hyperimmune sera including the negative control were also prepared as reported by Wesonga et al [6].

2.2. DRUG PREPARATION, SAMPLE COLLECTION AND SERUM PREPARATION AND CHEMICALS/REAGENTS

Animals were treated with CAP sodium succinate whole blood samples collected from which serum was prepared [6]. Carbonate–bicarbonate buffer phosphate Buffered Saline plus Tween 20 (PBST) Na_2HPO_4, M KH_2PO_4 and Tween 20 (PBST) dextran coated charcoal, Normal saline, Ammonium sulphatewere used [6].

2.3.CAP CONJUGATE, ANTI–CAP ANTIBODY AND PURIFICATION

The conjugation of CAP to horseradish peroxidase was achieved by adding CAP to N–(3–dimethylaminopropyl)–N–ethyl carbodiimide hydrochloride and HRP followed by purification [6]. Anti–CAP antibody raised in the 3 animals was purified by precipitation using ammonium sulphate and is detailed elsewhere and the optimal anti–CAP antibody and conjugate titres were determined by a checkerboard titration [6].

2.4. ELISA

The test samples were analysed for CAP by a competitive ELISA [6] and the drug concentrations of unknown samples were calculated using the Eiaquik program designed by Eisler [7] from a calibration standard curve fitted using a four–parameter logistic regression of CAP standards [6]. Pooled serums with optical densities within 2 standard deviations of the mean of the drug were used to prepare matrix matched calibration curves including serial dilutions of 250 ng/mL, 500 ng/mL and 1,000 ng/mL material [6].

2.5. METHOD OPTIMIZATION/VALIDATION

The cross–reactivity of CAP reagents with other commonly used antibiotics, such as penicillin, tetracycline and sulfamethazine was determined as the concentration of CAP which yields 50% inhibition (IC_{50}) with the amount of the competitor drug giving the same inhibition while the LOD was established by measuring the optical densities of 30 negative control serum samples where the LOD deviations (0.1 ng/mL) was the Mean OD minus two standards [6]. The limit of quantification (LOQ, 0.193 ng/mL)) was determined as equivalent to concentration to mean less 3 x the standard deviation [6].

Intra–assay and inter–assay variations were evaluated by analysing the 6h serum pool diluted in control serum at 1/300 and 1/3,000 and the method was validated using serum samples from sheep injected with CAP at the recommended dose of 25 mg/kg body weight [6].

3. RESULTS AND DISCUSSION

Purified antibodies from camels were used for the ELISA test as theyproduced higher optical densities than the unpurified camel antibody and purified [6]. The suitable concentrations of calibration standards from a 3–fold serial dilution were 500 ng/mL, 18.51 ng/mL, 6.17 ng/mL, 2.06 ng/mL, 0.68 ng/mL, 0.22 ng/mL, 0.07 ng/mL and 0.02 ng/mL; and the LOD 0.1 ng/mL, while the intra–assay (CVw and inter–assay variations (CVb) ranged from 2.62% to 9.64%. Parallelism was demonstrated in the different samples at various dilutions while no cross reactivity was observed with penicillins, tetracycline, sulfamethazine and streptomycin [6].

3.1. DETERMINATION OF CAP IN SERUM

Serum was collected from sheep treated with 25 mg/kg bw CAP and a mean (n=5) CAP levels 16 h and 7 days later wereof 2381±226 ng/mL (mean ±SE) and 0.21 ±0.06 (mean ±SE) ng/mL, respectively [6]. The method LOD of 0.1 ng/mL produced in this method [6] was significantly lower, than that reported elsewhere [8]. The method can therefore meet the requirement of the EU recommended minimum required performance limits (MRPL) of 0.3 ng/mL [9].

4. CONCLUSION

A sensitive and specific ELISA technique for analysis of CAP has been developed and validated. No cross–reactivity was observed between CAP and other commonly used veterinary drugs. The method is useful for pharmacokinetics and residues testing work and should be handy for countries with limited resources.

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CHLORAMPHENICOL IN SHEEP

WESONGAH, J.O[*]., MURILLA, G.A[*]., GUANTAI, A.N[**]., MDACHI, R.E[*]
[*] Kenya Agricultural Research Institute, Residue Analysis Section, Kikuyu, Kenya
[**] The University of Nairobi, College of Health Sciences, Faculty of Pharmacy, Department of Pharmacology and Pharmacognosy, Nairobi, Kenya

Abstract

The pharmacokinetics of chloramphenicol (CAP) sodium succinate was also studied in sheep following intramuscular injection of CAP. A CAP–ELISA technique was used and the mean maximum concentration (Cmax), the time taken to reach maximum concentration (t_{max}), the mean residence time (MRT) and mean elimination half–life calculated. The total body clearance (Cl_B) obtained was 0.00024 ± 0.000036 mL/h per kg, demonstrating prompt absorption and distribution of the drug following injection.

1. INTRODUCTION

The broad–spectrum antibiotic CAP produced by *Streptomyces venezuelae* [1, 2] is restricted to non–food producing animals. It interferes with protein synthesis of gram–negative and gram positive bacteria but is associated with toxic effects [3, 4] following various means of exposure [5, 6]. In the current study, a CAP–ELISA technique was used to study the pharmacokinetics due to better sensitity with a limit of detection (LOD) of 0.1 ng/mL compared to previous studies using other species [7–10].

2. MATERIALS AND METHODS

2.1. EXPERIMENTAL ANIMALS, CAP ADMINISTRATION AND SAMPLE (BLOOD AND SERUM) COLLECTION

As reported elsewhere [11], 8 red Kenyan maasai sheep (21 kg–25 kg) 9 to 12 months (m) old were included in the study.The animals were fed and watered ad lib and treated against ecto– and endo–parasites. Fresh aqueous CAP sodium succinate (10%, w/v; Nabros Pharma, India) was administered at a dose of 25 mg/kg body weight, by deep intramuscular injection into the right hind limb muscles of each animal [11]. Blood was collected and left to clot before storage at +4°C overnight and later centrifuged at 1,200g for 30 min to separate serum. The samples were collected at 5, 10, 15 and 30 min; 1, 2, 4, 6, 8, 12, 24 and 32 h; twice daily in the first week, then daily in week two, thrice in week three and lastly twice in the fourth week [11].

2.2. CAP ANALYSIS BY ELISA

Competetive ELISA was used with anti–CAP antibody raised in camel, phosphate buffered saline (PBS), Tween 20 and tetramethylbenzidine–hydrogen peroxide (TMB/H_2O_2) used as chromogen–substrate system and the reaction topped with orthophosphoric acid [11]. The LOD of 0.1 ng/mL was equivalent to the concentration corresponding to the mean OD of 30 negative control sera less two standard deviations [11]. The non compartment pharmacokinetics was conduced and using the WinNonlin® version 1.1 software package [12] pharmacokinetic parameters were as reported elsewhere [13]

3. RESULTS AND DISCUSSION

The mean serum CAP concentration was 0.26±0.03 ng/mL after a week but 0.1 ng/mL 2 days later [11]. The mean peak serum CAP concentration (C_{max}) was 134,132 ± 34,216 ng/mL at

the t_{max} of 0.18 ± 0.05 h, while the estimated mean apparent elimination half–life ($t_{1/2}\beta$) obtained in sheep was 36.4 ± 3.66 h. Other parameters are detailed in Table 1 [11].

The pharmacokinetic constants for CAP depend markedly on the drug formulation [14] and the sensitivity of the analytical method (e.g. LOD of 0.1 ng/mL) [8, 9, 11]. The elimination half–life of (36.4±3.66 h) obtained [11] was seven fold longer than in merino sheep treated intramuscularly with a dose of 50 mg/kg [15]. This difference in the half–life values could be attributed to the different breeds [11]. It has been observed [15] that serum concentration above 5,000 ng/mL were maintained for approximately 12 h post drug administration in sheep, however, similar concentrations could be only maintained for approximately 4 h post drug administration and this could be due to dose differences [11].

The observed mean residence time between sheep and goats [11] has also been reported before [16] though with involving different drugs yet CAP is known to have short mean residence time in sheep [17] following intramuscular administration. Al–Nazawi and Homeida [18] also reported a low body clearance rate of 0.018 mL/min/kg in camels treated intramuscularly with CAP at 5 mg/kg bodyweight. These findings indicate a similarity in clearance of CAP among species [11] while the low body clearance in sheep could be due to plasma protein binding [19–21]. Although the therapeutic plasma concentration of CAP is generally between 5,000 and 8,000 ng/mL [10, 22, 23], and indeed CAP concentrations above 8,000 ng/mL were only maintained for a couple of hours after administration [11].

TABLE 1. PHARMACOKINETIC PARAMETERS OF CAP SODIUM SUCCINATE IN INDIVIDUAL SHEEP TREATED INTRAMUSCULARLY AT 25 mg/kg BODYWEIGHT [11]

Parameter	Animal Number								Mean ± SE
	1	2	3	4	5	6	7	8	
Dosing time	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0 ± 0
Rsq	1.00	0.89	1.00	0.98	0.99	0.88	0.82	0.96	0.94 ± 0.02
T_{max} (h)	0.16	0.08	0.08	0.16	0.50	0.08	0.16	0.25	0.18 ± 0.05
C_{max} (ng/mL)	86050	28692	44640	133370	40210	312800	220730	206560	134132 ± 34216
Tlast	248	200	272	272	344	272	296	272	272 ± 13.4
Clast	0.05	0.23	0.15	0.16	0.02	0.22	0.02	0.17	0.13 ± 0.03
AUCo-last (ng.h/mL)	79168	58278	76720	108852	119132	186639	168103	198949	124480 ± 17810.7
β	0.03	0.02	0.02	0.02	0.04	0.02	0.02	0.01	0.02 ± 0.03
t1/2β (h)	23.18	43.22	39.27	34.47	17.10	44.13	39.64	49.94	36.37 ± 3.66
AUCo-∞ (observed) (ng.h/mL)	79170	58292	76728	108859	119133	186653	168104	198961	124488 ± 17811
AUCo-∞ (predicted) (ng.h/mL)	79170	58293	76728	108860	119133	186644	168106	198960	124487 ± 17810.4
AUMCo-last ($ng.h^2/mL$)	142603	176352	182053	275595	485478	390141	651675	535756	354957 ± 62773.4
AUMCo-∞ (observed) ($ng.h^2/mL$)	143074	180115	184846	278155	485660	394842	652079	539969	357343 ± 62679.7
AUMCo-∞ (predicted) ($ng.h^2/mL$)	14307	180511	184889	278253	485668	391978	652678	539658	340993 ± 70813.2
CL_B mL/h per kg	315.776	428.873	325.822	229.653	209.8495	133.9384	148.717	125.652	239 ± 203
MRTo-last (h)	1.80	3.03	2.37	2.53	4.08	2.09	3.88	2.69	2.81 ± 0.27
MRTo-∞ (observed) (h)	1.81	3.09	2.41	2.56	4.08	2.12	3.88	2.71	2.83 ± 0.27
MRTo-∞ (predicted) (h)	1.81	3.10	2.41	2.56	4.08	2.10	3.88	2.71	2.83 ± 0.27

3.1.CONCLUSION

The pharmacokinetic of CAP was studied in sheep and plasma levels determined by a sensitive analytical method. The drug levels were detectable within one week of administration.

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DEVELOPMENT AND VALIDATION OF AN IMMUNOASSAY KIT FOR THE SCREENING OF FLORFENICOL IN FISH TISSUE SAMPLES

GRANJA, R.,
Laboratorio Microbioticos, Campinas, Sao Paulo, BRAZIL

Abstract

Florfenicol (FFC) is an antibiotic used to treat bacterial diseases in fish. The corresponding Maximum Residue Limit (MRL) in fish muscle is 1, 000 μg/kg. A radioimmunoassay (RIA) technique has been developed, validated and used to screen residues of florfenicol and its amine analogue in fish muscle in Brazil. Confirmation was done using an LC–MS/MS.

The study included the development of a RIA kit for screening FFC in Fish tissue samples. Antibodies were manufactured by Reha–Biotech in Brazil and Queens University in Belfast (QUB).

The antibodies developed in Brazil were capable of detecting FFC and florfenicol–amine (FFA) at ~ quarter and 0.625 maximum recommended residue limit (MRL), respectively. The antibody developed by QUB was able to detect both FFC and FFA at a quarter FFC MRL.

Sample preparation involved extraction/clean–up of 5 g of fish tissue using acetone, water and dichloromethane-hexane (for defatting). The sample was then reconstituted in physiologic buffer before RIA. The method was validated according to Brazilian and European Union (EU) Guidelines and parameters such as CCα and CCβ, cross reaction in matrix, stability of standard solutions, and stability of the analyte in matrix as well as method uncertainty, studied. The CCα and CCβ levels were 100 μg/kg and 250 μg/kg, respectively. No cross reaction in matrix was not observed. There was also no statistical variation in the concentration of analytical standards in solution or matrix. Method uncertainty values were ~ 5.9% and 10.2% for FFC and FFA, respectively. The method is fast and reliable for monitoring residue levels of FFC in fish. Confirmatory assays by HPLC–UV and LC–MS/MS were also developed and implemented. A RIA kit was also developed for the determination of FFC in fish muscle.

1. INTRODUCTION

The thiamphenicol derivative, FFC is an antibacterial agent for human medicine important in risk management strategies for non–human use [1, 2]. It has also been identified by the AOAC International as an important molecule requiring the development of effective methods food safety such as in the seafood sector. The drug was prioritized by the Brazilian Residue Control Program in order to meet export and national requirements. Its MRL is 800 μg/kg. This project involved the development of a RIA kit for the screening of FFC a critically important antimicrobial in fish tissue samples.

The project scientific scope included method development, standardization and validation of a RIA kit and related analytical tool for the determination of FFC residues in fish tissues; Conduct an equivalence study of the validated method with other methods (such as HPLC and MS); applying the validated method to samples collected for the Official Monitoring Program in Brazil; participating on interlaboratory rounds conducted by Microbioticos and International Bodies.

2. MATERIALS AND METHODS

Tritium labeled FFC (specific Activity: 3 Ci/mmol; Radioactivity Purity ≥97%; 5,000 cpm/tube); FFC antibody (Supplier RBiotech–Brazil); Dilution 1:10,000 (lyophilized: 30 mg/150 tubes); FFC Standard (Calibration curve ranges from 250 μg/kg to 2,000 μg/kg).

The FFC standard was provided by Sigma–Aldrich, USA and radiolabelled FFC by Moravek Radiochemicals, USA with a specific activity of 3 Ci/mmol; radioactivity purity ≥97% and structure confirmed by Tritium Nuclear Magnetic Resonance. An antibody was developed and this was tested using tritium labeled CAP as marker; sixty fish samples were analysed using a developed HPLC–UV; equivalence study was of the HPLC–UV method was done against the LC–MS/MS technique.

The developed antibody for FFC in the RIA kit was tested against CAP tritium labeled compound. The combination of FFC antigen, FFC antibody and CAP marker showed a low sensitivity (perhaps due to the slope of calibration curve obtained). Thus a manufactured FFC tritium labeled compound was considered for better results.

Fifty one fish samples were analysed for FFC under the 2010 Brazilian National Residue Regulatory survey until February 2011. No samples presented violative results. Detailed Information on this survey is provided on Table 1.

TABLE 1. NUMBER OF SAMPLES ANALYZED PER BRAZILIAN STATE (PRODUCING AREAS) FOR FFC RESIDUES IN FISH MUSCLE SAMPLES – 2010 SURVEY

Brazilian State	Number of Samples Analysed	Quantified or Violative Results
MT	19	-
AL	1	-
BA	1	-
SP	18	-
SC	2	-
PR	3	-
MS	7	-

According to the EU decision 2002/657 [3] for permitted drugs such as FFC, an HPLC–UV technique and a separate chromatographic system or an independent detection method are appropriate for confirmatory purposes. In this study equivalence of the HPLC–UV detection to HPLC–DAD detection system was studied, with quantification on analytes based on a matrix calibration curve (in triplicate). A sensitive response was noted when using the second detection system and CCα, CCβ and Relative Standard Deviation (RSD) were determined (Table 2). Thus the HPLC–DAD system was used to support confirmatory tests in this study.

TABLE 2. COMPARISON ON CRITICAL METHOD INFORMATION USING TWO DIFFERENT DETECTION SYSTEMS

Method	CCα (µg/kg)	CCβ (µg/kg)	RSD (%)
HPLC–UV	840.27	879.45	3
HPLC–DAD	826.70	853.41	5

Given recent trends with MS as a means of confirmation in most regulatory areas, an equivalence study between HPLC–UV and LC–MS/MS was done in this study. The extracts obtained from the clean–up were injected on an LC–MS/MS detection system with separation on a Zorbax 150×2.1×5 µm column using alkaline H_2O:MeCN mixture as the mobile phase. A sensitive response was noted with the Mass Spectrometric System, comparable to the HPLC–UV system and the CCα, CCβ and RSD obtained (Table 3). The LC–MS/MS system corroborated results obtained when using the HPLC–UV/DAD. Thus an earlier developed standard operating procedure (SOP, NS 5.04–119–LB) [4] was revised accordingly to include both the HPLC–DAD and LC–MS/MS system as possible confirmatory methods.

TABLE 3. CCα AND CCβ WHEN USING LC–MS/MS (COMPARED TO HPLC METHOD)

Method	CCα (μg/kg)	CCβ (μg/kg)	RSD (%)
LC–MS/MS	829.30	861.40	5.4

Antibodies produced in Brazil were also compared with those provided by Queen´s University Belfast (QUB) and the Brazilian Antibody validated *visa vis* CAP Marker system according to decision 2002/657/EC [3]. The validation included experiments on the CCα and CCβ; cross reaction in matrix; stability of standard solutions; stability of analyte in matrix. The Brazilian antibody was initially purified with protein G affinity chromatography using acetate buffer 0.1M pH 5.0 as column washing buffer and Tris–Glicine 0.1M pH 2.8 as elution buffer.

Elution fractions were measured using a spectrophotometer at λ=280 nm and fractions presented Optical Density greater than 1.0 gathered and concentrated using a Amicon Ultra–15 (Millipore) system until a final purified immunoglobulin concentration of 3 mg/mL been reached. The produced antibodies were then evaluated by means of indirect ELISA using conjugated FFC with KLH at 10 μg/mL to 0.125 μg/mL and using KLH at 5 μg/mL as control. The Chromogen–Peroxidase reaction Reading (OPD) was satisfactory and showed positive reaction at optical density of 0.250 μg/mL less so at optical density of 0.125 μg/mL). The indirect ELISA test results were close to the range 200 μg/L–2,000 μg/L. It was expected that FFC molecules binds to the agarose via Cyanogen Bromide. Thus following purification, the antibody needed to be charged onto the column in such a way to promote the antigen-antibody binding to the column, eliminating the nonspecific immunoglobulin present in the original serum or immunoglobulin. The reaction between Tritium labelled FFC and QUB antibody was thereafter initiated.

2.1.1. Validation of the system involving Brazilian antibody with CAP as Marker

The Brazilian antibody was tested against CAP tritium labeled compound. The sensitivity was however less than expected although since FFC MRL in Brazil is 1,000 μg/kg for fish, the detection limit was good enough that the method could still be used for screening purposes. Validation followed European Commission Decision 2002/657 using a previously generated a protocol in house [5].

The procedure included work on CCα and CCβ, where CCα was determined by analysing 20 blank fish muscle samples; the mean signal plus three times the standard deviation was the CCα. The CCβ was also determined by analysing series of blank tissue spiked with different levels of florfenicol (150 μg/kg to 2,000 μg/kg) with a tolerable false negative rate of 5%. The minimum concentration with lower than 5% rate was the CCβ. Cross reaction in matrix was also studied using analytical calibration curves and matrix calibration curves. The two curves were compared and the corresponding “signal–ratio” value reported as percentages.

For the stability of standard solutions, statistical analysis (t–test) was performed on routine calibration curves used for analysing batches of FFC in routine fish muscle residue analysis (using validated and accredited HPLC–UV method). The stability of analytes in matrix was investigated by a spiking matrix at 5 times the CCβ levels using two sets of sample replicates (n=5).These were then analysed after 15, 30 and 45 days of storage. The maximum sample turnaround time allowed by Brazil Ministry of Agriculture is 15 business days, while the EU

allows a 6 weeks' time frame for receipt of analytical report after sample is recorded in the laboratory. The validation results are expressed in Table 4.

TABLE 4. RESULTS OBTAINED FOR METHOD VALIDATION USING FLORFENICOL ANTIBODY AND TRITIUM LABELED CAP

Parameter	Result	Remarks
CCα	~60 µg/kg	-
CCβ	~250 µg/kg	-
Cross Reaction–Matrix	~32%	The relevance of this information should be addressed because the decision if a sample is compliant or not is based on a comparison between the sample result and the spiked samples at MRL.
Stability of Standard Solutions	Ok	Student t–test criteria was been fulfilled according to the validation reference*
Stability of Analyte in Matrix	Ok	No variation was observed on analyte concentration according to the validation reference*

* Granja et. al [5]

2.1.1.1.Method application

Fifty two fish samples were analysed for FFC under the Brazilian National Residue Regulatory survey (Table 5).

TABLE 5. NUMBER OF SAMPLES ANALYZED PER BRAZILIAN STATE (PRODUCING AREAS) FOR FFC RESIDUES IN FISH MUSCLE SAMPLES IN A 2011 SURVEY

Brazilian State	Number of samples analysed	Quantified or Violative results
Mato Grosso do Sul (MS)	2	-
Mato Grosso (MT)	18	-
Pernambuco (PE)	3	-
Paraná (PR)	16	-
Santa Catarina (SC)	2	-
São Paulo (SP)	7	-
Tocantins (TO)	4	-

A study was also conducted using Agarose–Cyanogen Bromide chromatography to increase the FFC antibody specificity. A FFA antibody was also developed and tested against QUB and validation was further done according to EC decision 2002/657/EC [3]. The antibodies developed in Brazil were found to be capable of detecting FFC and FFA (the major metabolite and also a marker residue) at almost a quarter MRL (like the antibody developed by QUB) and 0.625 MRL, respectively.

2.1.2. Sample preparation for RIA and method validation

In the clean–up method 5 g of fish tissue were extracted in acetone, water and defatted with dichloromethane–hexane. The sample was then reconstituted with physiologic buffer and the submitted for RIA. The method was validated according to Brazilian and European Union Guidelines and parameters such as CCα and CCβ, cross reaction in matrix, stability of standard solutions and stability of the analyte in matrix, determined. Method measurement uncertainty was also estimated.

The CCα and CCβ values were ~100 µg/kg and 250 µg/kg, respectively. Cross reactivity in matrix was not observed, and no differences were noted on the stability of the analytical standards when prepared in solution or matrix. Method uncertainty was around 5.9% and 10.2% for FFC and FFA, respectively. The method was found to be effective as a fast and reliable way to screen/monitor residue levels of FFC in fish. Also a RIA kit was developed for the determination of FFC in fish muscle.

2.1.3. Development of Antibody in Brazil and comparison with QUB antibody

Tritium labeled FFC was synthesized at Moravek Radiochemicals–Brea, California, and FFC. Initially the antibodies produced in Brazil and those provided by QUB showed no competition between FFC (labeled and non–labeled). This was proven to be due to a wrong antibody dilution at 1:20,000 where competition between radiolabelled and non–labeled was not possible. A corrected dilution of 1:1,000 to 1:2,500 was then found to be the optimal concentration promoting competition between radiolabelled and non–labeled).

The Brazilian antibody was initially purified with protein G affinity chromatography using Acetate Buffer 0.1M pH 5.0 as washing column buffer and Tris–Glycine 0.1M pH 2.8 as elution buffer. Elution fractions were measured using a spectrophotometer at λ=280 nm and fractions with Optical Density greater than 1.0 gathered and concentrated using a Amicon Ultra–15 (Millipore) system until a final purified immunoglobulin concentration of 3 mg/mL. The produced antibodies were evaluated by means of indirect ELISA using conjugated FFC with KLH at 10 µg/mL to 0.125 µg/mL and KLH at 5 µg/mL as a control. The Chromogen-Peroxidase reaction reading showed a positive reaction at optical density of 0.250 µg/mL, but less so at optical density of 0.125 µg/mL.

The FFC detection range was 200 µg/L–2,000 µg/L levels, and the indirect ELISA levels obtained were very close to this. A FFC antibody specific adsorption procedure using Agarose–Cyanogen Bromide chromatography was thus conducted to enhance the method performance. We expected that if the FFC molecule bound to the agarose via Cyanogen Bromide and following purification of the antibody, it would adhere onto the column so as to promote the antigen–antibody binding to the column, and thus eliminating the nonspecific immunoglobulin present in the original serum or immunoglobulin from the immunized animal.

This technique aimed at raising the sensitivity of the antibody solution and the result was a 0.312 µg/mL detection level. The ideal dilution was 1:10,000 and a reaction against tritium labeled FFC was observed.

2.1.4. Brazilian antibody–FFC tritium labeled as Marker

The re–purified Brazilian antibody was re–tested against FFC tritium labeled compound developed. The sensitivity at a dilution of 1:10,000 was ideal for Brazil's MRL of 1,000 µg/kg in fish and therefore supported the national residue monitoring program.

2.1.5. Development of a FFA antibody

Since FFC residues are expressed as the sum of FFC and FFA, it is important to monitor both substances ideally in the same test. Because the FFC antibody did not show a reaction with the FFA standard when tritium labeled FFC was used as a marker (cross reaction of less than 1%), a study to develop anti FFA antibody was conducted.

2.1.6. Immunization Scheme

Two New Zealand rabbits were inoculated subcutaneously with three doses and one booster, the first dose with Freund Adjuvant and incomplete adjuvant on the subsequent doses (Table 6*)

TABLE 6. IMMUNIZATION SCHEME FOR FFA AMINE ANTIBODY PRODUCTION

Day	Dose	Amount	Adjuvant
0	First	200 µg	Complete
11	Second	300 µg	Incomplete
21	Third	400 µg	Incomplete
28	Proof bleeding	-	-
30	Booster	400 µg	Incomplete
37	Blank bleeding	-	-

(*) The experiments have been carried out following all Brazilian experience animal ethic procedures

2.1.6.1.Proof bleeding and reactivity/quality tests

Five mililitre of blood was collected through a heart puncture (proof bleeding) followed by blank bleeding where 50 mL of blood was collected by heart puncture to obtain the polyclonal antibody. The polyclonal antibody obtained was been titrated and the antisera evaluated using indirect ELISA as applied elsewhere [6]. The ELISA assays demonstrated a positive response for the antigen (from proof bleeding) and it was significantly enhanced because of the booster dose and subsequent blank bleeding. The polyclonal antibody dilution was found to be around 1:10,000. The antigen used was FFA conjugated with BSA in order to drive the antigen–antibody response to only this two species and avoiding responses associated with KLH used as carrier protein during immunization.

A KLH protein was used as a control to evaluate the antibody response against the complex FFA–KLH in comparison to the FFA response. The results obtained demonstrated that the polyclonal antiserum responded to FFA and the immunization was successful.

The minimum concentration at which the antibody recognizes the antigen was also evaluated. The indirect ELISA demonstrated the sensitivity the concentration of 0.625 µg/mL of antigen using dilutions of 1:10,000 or 1:5,000.

2.1.7. Validation of QUB–antibody system–tritium labeled FFC as marker

The new dilution of the QUB antibody for FFA showed a strong reaction against marker and analyte, with good competition between binding sites. The Brazilian antibody system could detect both FFC and FFA in the same run. This facilitated the development and validation of the analytical method in order to detect FFC and FFA residues in fish tissues. Liquid–liquid extraction was preferred to SPE to cut costs.

2.1.7.1.Extraction and clean–up procedure prior to RIA

The procedure included:

(1) Weighing 5 g homogenized tissue into 50 mL graduated screw–cap polypropylene centrifuge tube
(2) Fortifying with aliquot of working standard at this stage and adding 2 mL deionized H_2O to tube
(3) Adding 8 mL acetone and homogenizing contents

(4) Centrifuging at 1,500 rpm for 10 min and adjusting final volume of combined supernatant, precipitating to 20 mL with acetone, and decanting supernatant into a tube
(5) Mixing supernatant well, transfering 8 mL to a screw–top tube, and adding 6 mL dichloromethane
(6) Capping the tube and mixing contents with vortex mixer (5 sec)
(7) Centrifuging for 5 min at 1,000 rpm to separate the layers
(8) Drawing off and discarding upper aqueous layer, and evaporating lower dichloromethane layer to dryness using nitrogen (maximum temperature, 45ºC)
(9) Dissolving residue in 1 mL 0.1% acetic acid solution and extracting with 2 mL hexane
(10) Centrifuging at 1,000 rpm for 5 min to allow layers to separate, and drawing off and discarding upper hexane layer
(11) Taking 250 µL and completing to 500 µL with physiologic buffer.
(12) Completing RIA

RIA including

(1) Preparing calibration points at a final concentration range from 250 µg/kg to 2,000 µg/kg using physiological buffer (pH = 7.4) as solvent.
(2) Adding 5,000 cpm to 8,000 cpm of tritium labeled FFC
(3) Adding 100 µL of antisera at a dilution of 1:2,500 to each tube
(4) Incubating 15 min at 37ºC
(5) Incubating overnight at 4ºC.
(6) Adding 1 mL of charcoal (0.5%) dextran (0.05%)
(7) Leeting content to absorp for 10 min at 4ºC
(8) Centrifuging at 10,000 rpm
(9) Removing the aqueous layer
(10) Adding 5 mL of scintillation cocktail
(11) Reading samples for 4 min using a scintillation counter

The RIA technique was validated according to European Union 2002/657 decision [3] on the following parameters, following the protocol developed elsewhere [5].

2.1.7.2.CCα and CCβ

(1) CCα was determined by analyzing 20 blank fish muscle samples. The mean signal plus three times the standard deviation was the CCα.
CCβ was also determined by analyzing series of blank tissue spiked with (250 µg/kg). Only one false negative sample was acceptable.

2.1.7.3.Cross reaction; Stability of standard solutions and matrix

Since the blank fish muscle samples had no interferences that would affect the FFC signal/response, we assumed that cross reaction in matrix would not be observed.

For the stability of standard solutions and matrix, Shewhart statistical test analysis was conducted on previous data obtained from analytical curves during routine residue analysis of florfenicol in fish muscle; a validated and accredited HPLC–UV method was used. The

quality control chart demonstrated that the method's performance was under control over a long period (>1 year). We therefore made an assumption that standard solutions could be stable for more than one year when stored at 4ºC–9ºC.

Study of analyte stablity in matrix was done by spiking a set of sample replicates (n=5, at CCβ levels) that were stored and analysed after 45 days. The maximum sample turnaround time allowed by Brazilian Ministry of Agriculture is 15 business days while the European Union allows a 6 weeks' timeframe to the analysis report after sample receipt.

2.1.7.4.Method Uncertainty

This followed the rule of average of the sum of relative standard deviation squares obtained on the measured values. The whole method was investigated including weighting, sample preparation, preparation of solutions, following normal, triangular and rectangular distributions.

3. RESULTS AND DISCUSSION

In addition to the findings in Tables 3 and 4, the following were also observed on the method's suitability (Tables 7 and 8) and parameters of the method used (Table 9).

TABLE 7. METHOD VALIDATION DATA USING FFC AS ANALYTE, FFC QUB ANTIBODY AND TRITIUM LABELED FFC

Parameter	Result	Remarks
CCα	~ 100 µg/kg	-
CCβ	~ 250 µg/kg	The study didn´t go lower than that because this represents a quarter of the MRL. Brazilian authorities recommend when analyte content is sum of more than one compound the method should be able to detect half the MRL
Cross Reaction – Matrix	Not observed	-
Stability of Standard Solutions	Ok	Shewhart test criteria had been fulfilled according to the a previous validation protocol *
Stability of Analyte in Matrix	Ok	No visible variation observed on analyte concentration according to the validation reference*
Method Uncertainty	5.9%	

TABLE 8. METHOD VALIDATION USING FFA AS ANALYTE, FFC QUB ANTIBODY AND TRITIUM LABELED FFC

Parameter	**Result**	**Remarks**
CCα	~ 50 µg/kg	-
CCβ	~ 250 µg/kg	The study didn´t go lower than that because this level which represents a quarter of the MRL. Brazilian authorities recommend that when analyte content is the sum of more than one compound then the method should be able to detect half the MRL
Cross Reaction – Matrix	Not observed	-
Stability of Standard Solutions	Ok	Shewhart test criteria had earlier been fulfilled based on a previous validation protocol*
Stability of Analyte in Matrix	Ok	No visible variation in analyte concentration was observed when following a previous validation protocol*
Method Uncertainty	10.2%	

* Granja et. al; [5]

TABLE 9. MASS SPECTROMETRY CONDITIONS FOR FFA

Parameter	Setting
Ion Source	Atmospheric pressure chemical ionization (APCI, positive mode)
Corona	2 μA
Cone	25 V
Extractor	5 V
RF Lens	0.2 V
Source Temperature	130ºC
APCI Probe Temperature	500ºC
Desolvation Gas Flow	300 L/h
Cone Gas Flow	100 L/h

*Conditions for FFC are expressed elsewhere [7].

4. CONCLUSION

A Brazilian antibody against FFC was produced and successfully tested when applied to RIA test, and a RIA method validated. A FFA antibody was also successfully produced although no reaction was observed with tritium labeled FFC. Both antibodies were applicable to FFC monitoring purposes since they were able to detect FFC at a quarter MRL and FFA at almost half the MRL. This is acceptable to Brazilian regulations. The FFA antibody obtained could be applied to an ELISA competition ELISA using a pnPP or OPD as marker and read at 405 nm or 492 nm, respectively.

The antibody produced by QUB was successfully applied to a RIA system and could detect both FFC and FFA. The method was validated following Brazilian and EU Guidelines. A simple, reliable and cheaper clean–up method (excluding the use of solid phase extraction cartridges) was developed to support the residue monitoring program in Brazil.

A RIA kit for the determination of FFC and FFA could be setup following this method. Confirmatory methods based on HPLC–UV and LC–MS/MS were also developed.

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RADIOIMMUNOASSAY SCREENING METHOD FOR ANILINIC AND PHENOLIC BETA–AGONISTS IN BOVINE LIVER

GRANJA, R. H. *., MONTES NIÑO, A. M *,., RABONE, F*., MONTES NIÑO, R.E*., CANNAVAN, A**, SALERNO, A.G*
* Microbioticos Analises Laboratoriais, Campinas, Sao Paulo, Brazil
** International Atomic Energy Agency

Abstract

The screening method of selected β_2–agonists in bovine liver was studied following European Union (EU) Commission Decision 2002/657/EC criteria. The detection capability (CCβ), ranged from 0.25 μg/kg to 0.5 μg/kg. The technique was found applicable to routine monitoring program.

1. INTRODUCTION

Beta agonists are commonly used to treat pulmonary conditions in some cases as growth promoters [1]. The latter is prohibited in maney countries such as the European Union (EU) according to Council Directive 96/22/EC [2]. Bronchodilators such as clenbuterol and mabuterol are β_2–agonists bronchodilators used to treat chronic and obstructive pulmonary diseases in animals and humans [3, 4]. Orally administered clenbuterol possibly the most common aniline type β_2–agonists, is absorbed by liver, lungs, kidneys and some secretory organs [5–8] with concentrations decreasing rapidly except in the liver, retina, hair and feathers that are therefore suitable study animal matrices [9, 10]. Another β_2–agonist mabuterol is more gradually eliminated [11, 12]. As reviewed elsewhere [13] like other β_2–agonists clenbuterol and mabuterol have been used in animal production to improve carcass quality [13].

According Granja et al [13] reliable, easy to use and cost effective multiressidue tecehniques are necessary given that several banned β_2–agonists substances are commonly used and may be available in trace amounts. Techniques such as surface plasma resonance biosensor assay [14], enzyme–linked immunosorbent assay (ELISA) has been described for β_2–agonists in hair [15–17] have been reported though some drawbacks [19, 20].The radioimmunoassay (RIA) is a suitable alternative with excellent sensitivity, specificity and method performance for a multiresidue screening method [21, 22]. As reported elsewhere [13] the immunological techniques have been applied to monitor β-agonists such as clenbuterol and salbutamol in Brazil [13].

Therefore the aim of this study was to develop a general strategy to screen β_2–agonists in bovine liver samples using RIA. As reported by Granja et al [13] an analytical method for monitoring mabuterol residues in bovine liver samples had been developed prior followed by method validation as outlined elsewhere [23].

2. MATERIALS AND METHODS

2.1.REAGENTS AND CHEMICALS

Analytical grade materials were obtained from Merck (Darmstadt, Germany) and Carlo Erba (Milano, Italy); phosphate buffer (pH = 6.8), phosphate buffer PBS–gelatin (pH = 7.4), acetate buffer (pH = 5.2) and Trizma base buffer (pH = 9.1); H_2O from a Milli–Q Gradient System A–10 (Millipore, Billerica, MA, USA); Solid phase extraction (SPE, Bond Elut C–18 cartridge;Varian (Palo Alta, CA, USA). β–Glucuronidase (Helix pomatia, pH = 5.3);

Salbutamol (Sigma Aldrich, Saint Louis, MO, USA). Cimaterol, brombuterol, cimbuterol, clenproperol, and mapenterol (Witega, GmbH, Germany); ^{3}H clenbuterol (Laboratorie d´Hormonologie, Belgium); polyclonal antibody (Chemical Surveillance Department, Agri-Food and Biosciences Institute, Belfast, Northern Ireland).

2.2.APPARATUS

The following were employed: refrigerated centrifuge (Revan Cycle CR), liquid scintillation counter (Beckman LS 6000 TA), TecVap TE–0194 sample evaporation system, a nitrogen evaporation system, vacuum manifolds (Baker); incubator (Olidef CZ).

2.3.EXTRACTION OF ANILINIC BETA–AGONISTS FROM LIVER SAMPLES

Liver samples weighing 0.5 g were transferred into a 15 mL centrifuge tube and homogenized with 5 mL of a phosphate buffer (pH 6.8)–methanol (MeOH) mixture (95:5, v/v) followed by liquid–liquid extraction using 2 mL of MeOH, 3 mL of chloroform and centrifugation (1,560g, 4°C, 10 min) [13]. The supernatant was transferred to another tube, sodium hydroxide (0.7 mL) added, and extraction with diethyl ether (5 mL) done. The upper (ether) layer was gently evaporated to dryness at 37°C using nitrogen followed by quantification by RIA [13].

2.4.EXTRACTION OF SALBUTAMOL FROM LIVER SAMPLES

As reported elsehwre [13] 1.0 g of liver was weighed into a 15 mL falcon tube, and the sample hydrolyzed for 2 h at 37°C with acetate buffer and β–glucuronidase (200 U) followed by ultra–sonication for 15 min and centrifugation (1,560g, 4°C) for 10 min. The supernatant was transferred to a 15 mL falcon tube, 5.5 mL of trizma base buffer added and the sample cleaned on C18 SPE cartridges preconditioned with trizma base buffer, MeOH and H_2O. The SPE column was rinsed with a H_2O/acetonitrile (MeCN) mixture and eluted with a MeOH: phosphate buffer (99.5 mL: 0.5 mL) mixture. The organic phase was then evaporated at 60°C using nitrogen and the analyte (salbutamol) detected by RIA [13].

2.5.RADIOIMMUNOASSAY

The standard curve was preparing by adding 0.1 mL aliquots of clenbuterol standard solutions in phosphate buffer (pH 7.4; 0.01 M with 0.01% sodium azide) to RIA tubes equivalent to 0, 7.8 pg, 15.6 pg, 31.3 pg, 62.5 pg, 125 pg, 250 pg, 500 pg and 1,000 pg. Liver extracts (0.3 mL) and 0.1 mL of tritiated clenbuterol solution (3,500 cpm) were added to RIA tubes. In addition, antiserum (0.1 mL) was added to all but non-specific binding quantification tubes, the tubes vortexed and incubated overnight (4°C) before addition of dextran–coated charcoal mixture (0.5%, 0.5 mL) and incubation for 10 min at 4°C. The tubes were then centrifuged (1,560g, 10 min, 4°C) and 0.5 mL of the supernatant transferred into scintillation vials. Radioactivity was determined on a scintillation counter and the concentration calculated by comparison with the calibration curve [13].

2.6.METHOD VALIDATION

Blank liver matrix of 20 pooled proven as residue free (no analyte signal) were used.

The stability of the standard solutions was evaluated using a standard curve, at various intervals namely, 0 m, 3 m, and 9 m following initial preparation [13, 24]. Meanwhile, the stability of the analyte in matrix (at 4×CCβ) was monitored for 3 m of storage in a refrigerator.

The CCα was estimated by analysing 20 blank Brazilian bovine liver samples and the mean of the signal values plus three times the standard deviation was the CCα. The CCβ value was determined by fortifying 20 blank Brazilian bovine liver samples with low levels of each β_2–agonist (0.15 µg/kg, 0.2 µg/kg, 0.25 µg/kg, 0.3 µg/kg, 0.35 µg/kg, 0.4 µg/kg, 0.5 µg/kg) and the CCβ value determined as the lowest content of β_2–agonist detected in the samples with an acceptable false negative rate of 5% [13]. The antibody cross reactivity with a range of β agonists and specificity were also determined as reported elsewhere [13].

2.7.METHOD UNCERTAINTY

The measurement uncertainty was determined as reported elsewhere [25] based on a top-down approach also as recommended elsewhere [26]. All the β_2–agonists were included with samples spiked at 3 equidistant levels and the combined uncertainty measured [13].

3. RESULTS AND DISCUSSION

Cross–reactivity of the β_2–agonist antibody was exhibited for salbutamol, cimbuterol, clenbuterol, brombuterol, clenpenterol, mabuterol, mapenterol and cimaterol and therefore the antibody could be effectively determine these analytes. However, the method would only be useful as a qualitative or at best sem–quantitative manner in some cases since the cross reactivity was less than 100% for some of the compounds [13].

With the exception of salbutamol (often in glucuronidated form), pre–treatmenet and extraction of samples would be required for some analytes [13] It is nevertheless possible to analyse phenolic β_2–agonists A deconjugation step is preferered before clean–up and analysis [27] Additional modifications such as addition of basic solutions and enzymatic hydrolysis may be required [13] to achieve acceptable results during method validation using the 2002/657/EC [23]. The CCα, CCβ, and uncertainty estimates for the β_2–agonists were in the range 0.03–1.0 (µg/kg), 0.25–0.5 (µg/kg) and 4.58–877 (%), respectively [13] of mabuterol, cimaterol, mapenterol and sabutamol. Brombuterol, cimbuterol and clenproperol standard solutions were found to stable for no less than 9 m [13]. All the analytes namely clenbuterol, mabuterol, cimaterol, brombuterol, cimbuterol, clenproperol, mapenterol and salbutabol were stable in liver matrix over a 3 m period [13] when monitored according to Decision 2002/657/EC [23].

4. CONCLUSION

A robust RIA technique for screening a number of analinic and phenolic β_2–agonists in animal samples was developed and it is suitable for application to monitoring of the analytes in beef in Member States such as in Brazil.

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SYNTHESIS AND TRANSFER OF KEY REAGENTS FOR AN ^{125}IODINE CHLORAMPHENICOL RADIOIMMUNOASSAY

LANGE, I., HARTEL, A., REITER, M
Physiology Weihenstephan, Technical University Munich, Freising–Weihenstephan, Germany

Abstract

In recent years, several reports of Chloramphenicol (CAP) detected in poultry, aquaculture products or honey revealed that abuse of this antibiotic is still a serious problem, especially in developing countries. To identify and curb veterinary drug misuse, national food surveillance authorities have a great need for sensitive and efficient but non–sophisticated and low–cost screening methods. In this context, a ^{125}I solid–phase radioimmunoassay (RIA) for CAP was developed. ^{125}I as a γ–emmitter has the advantage of being directly detectable and the short half–life reduces problems with disposal of radioactive waste.

The competitive assay was conceived as a double antibody tube test with anti-rabbit coated tubes, specific anti-CAP antibody from rabbit and biotinylated CAP plus ^{125}I–labelled streptavidin as tracer. As ^{125}I–labelled CAP is not commercially available and direct labelling of CAP is rather labour–intensive and needs experience, aspects which limit the application in standard surveillance labs for which the test should be fit, ^{125}I–labelled streptavidin, which is commercially available and is easier to label was selected as tracer. The carboxyl group of the CAP metabolite, CAP succinate, provided a functional group for forming a peptide bond with 5–biotinamidopentylamine by carbodiimide coupling. Biotinylated CAP succinate was chromatographically purified and checked for reactivity to streptavidin in a modified enzyme linked version of the original RIA with streptavidin–horse radish peroxidise (HRP) as the label.

The synthesized product appeared to be biotinylated CAP succinate, judging by its UV absorption at 278 nm and binding both to anti–CAP antibody and streptavidin–HRP. The sensitivity of the tested standard curves showed that the tracer was fit for purpose. A new concept of ^{125}I solid–phase RIA was developed. It is applicable to a broad array of analytes and after adopting this technique to all relevant tests one lab needs only one radioactive label for all tests, making analytical procedures much easier.

All stages for the development of a practical and sensitive ^{125}I solid–phase RIA for CAP namely assay design, synthesis of biotinylated CAP and the first test on a modified enzyme–linked base, were elaborated,. The antibodies were stabilized and all reagents safely transferred to the project partners, who were additionally provided with assay protocols for the first tests to ensure establishment of the assay.

1. INTRODUCTION

Application of chloramphernicol (CAP) to food producing animals is forbidden within the European Union by Council Directive 2377/90 EEC appendix 4 [1]. In the US extra–label use of CAP in food–producing animals is prohibited by 21 CFR Ch. I § 530.41 [2]. Recently, several reports of positive residue testing of import products (aquaculture products, honey among others) revealed that CAP abuse is still a serious problem. To prevent export fluxes from being stopped because of health risks food surveillance authorities of exporting countries have a great need for sensitive and efficient screening methods to reveal and diminish drug abuse.

The Joint FAO/IAEA Division of the IAEA has been supporting national authorities in developing countries in residue monitoring for years. ^{125}Iodine based techniques are part of those projects. Rapid decay of this isotope, and therefore limited problems with disposal of radioactive waste, is one benefit. Additionally, ^{125}I as a γ–radiation emitter can be detected directly without adding any luminescent, unlike ^{3}H–labels, making laboratory procedures easier and cheaper.

The scope of this project, technical contract GFR 12332 under the IAEA coordinated research project (CRP) "Development of strategies for the effective monitoring of veterinary drug residues in livestock and livestock products in developing countries", was to develop a sensitive ^{125}I solid–phase radioimmunoassay for CAP.

This work was planned as a co–operation with the CRP research contract number TUR 11891 of the Animal Department of the Ankara Nuclear Research Centre in Agriculture and Animal Science of the Turkish Atomic Energy Agency. The task of the Institute of Physiology of the Technical University Munich–Weihenstephan was to design a solid–phase CAP ^{125}I–RIA, including preliminary experiments reported at the 1st Research Co–ordination Meeting in September 2002 in Vienna, synthesis and pre–checking of key reagents, transfer to the contract holder in Turkey and supervision of the development of the test. However, due to unforeseen circumstances, the test system could not successfully be established by the research group in Turkey. The protocols for the method were therefore transferred to another CRP research contract holder in Brazil for implementation in the future. A pre–requisite of the assay design was that as many reagents as possible should be easily commercially available or easy to synthesize.

2. MATERIALS AND METHODS

2.1. REAGENTS AND CHEMICALS

The specific anti–CAP antibody was kindly provided by Dr. Chris Elliott, Queens University, Belfast, UK, and Terry Fodey, Veterinary Science Division, Belfast, UK. All solvents used were of HPLC grade and reagents and chemicals were of analytical grade, unless otherwise stated.

2.2. FIRST SYNTHESIS OF BIOTINYLATED CAP SUCCINATE (2002/2003)

Following a procedure described by Meyer et al. [3] 68.75 mg CAP succinate sodium salt (Sigma C3787) and 18.13 mg N–hydroxysuccinimide (Aldrich 13 067–2) were dissolved in 2 mL dimethylformamide (DMF) (Sigma D–4254) and incubated with 31.25 mg N,N'–dicyclohexylcarbodiimide (Aldrich D 8000–2) in 2 mL DMF overnight at room temperature under gentle agitation. The mixture was poured over a porous frit and washed with 4×250 µL DMF. 50 mg 5–(biotinamido) pentylamine (Pierce 21345) dissolved in 1.7 mL purified water (H_2O) were added to the eluate and incubated for 4 h under gentle agitation (precipitate formed). The mixture was transferred into a 25 mL volumetric flask, which was filled to the mark with methanol (MeOH).

The product was diluted 1:10 (later 1:5; the product of the synthesis was not stable; the isolated product, however, appeared to be stable) with 30% aqueous MeOH and 250 µL aliquots were applied onto a RP18–HPLC column (PRONTOSIL®, 250×4.0 mm; Bischoff, Leonberg, Germany), which was protected by a RP18 cartridge (Phenomenex, Torrance, USA). The mobile phase selected was MeOH:H_2O (50/50, v/v). Fractions (500 µL) were collected (retention time 14–16 min) and tested in a newly designed enzyme immunoassay (EIA) protocol with streptavidin–HRP (Roche Nr. 1089153) as ^{125}I–labelled streptavidin surrogate. This replacement was made in order to avoid acquisition of ^{125}I–labelled streptavidin only for testing of the synthesized product.

Fractions were re–dissolved in purified H_2O and tested for simultaneous binding to anti–CAP antiserum and streptavidin–HRP: 100 µL diluted HPLC fractions and 100 µL of anti-CAP antiserum (code R684; 1:160,000; supplied by Prof. C. Elliott, QUB, Belfast) were incubated

over night at 4°C in anti–rabbit IgG coated microtitre plates (1 µg IgG/well; blocked with BSA). To determine the optimum antibody titre, in the first assay the antibody was titrated keeping label concentration constant. Later on antibody dilution was kept at 1:160,000 as described above. After decanting and washing the next day the plates were incubated with streptavidin–HRP (1:10,000) for 15 min at 4°C. After decanting and washing tetramethylbenzidine colour reaction was performed at 25°C for 40 min (standard enzyme immunoassay protocol according to [4]).

The tested conditions were kept constant and large–scale clean–up was started (249 injections). At the end of each day, the fractions containing biotinylated CAP succinate (identified by positive enzyme immunoassay signal) were combined and evaporated to dryness. When fractions of injection 1 to 50 were combined, evaporated and re–dissolved in MeOH, it appeared that this procedure led to a complete loss of reactivity (results not shown). Therefore the following fractions were re–dissolved in purified H_2O.

During large–scale purification product fractions of selected test samples were re–tested for quality assurance. After some weeks biotinylated CAP succinate appeared to be unstable in the synthesis mixture. The CAP peak grew in contrast to the product peak, which became smaller. This should be taken into account for future tracer synthesis.

The exact content of biotinylated CAP succinate was not quantified, as it was very difficult to evaporate the solvent and get sufficient dry residue to weigh. In addition, information on the exact content of tracer is not necessary for the assay protocol. Adequate dilutions have to be tested for every lot.

2.3. TRANSFER OF KEY REAGENTS TO THE TURKISH PROJECT PARTNER

For stabilization, first (anti–rabbit antibody) and second (anti–CAP) antibody were precipitated with ammonia sulphate following a standard operation protocol of the Institute of Physiology. Briefly, 158.2 g ammonia sulphate were added to 187 mL sheep–anti rabbit IgG solution (2 mg/mL) step by step. After precipitation of protein 374 mL H_2O were added. Bovine serum albumin (200 mg) was dissolved in 4.8 mL assay buffer (40mM NaH_2PO_4/Na_2HPO_4, 150mM NaCl, pH 7.2, 0.1% bovine serum albumin) and antiserum (0.2 mL) R684 was added. Protein was precipitated stepwise by adding 15 mL of saturated ammonia sulphate solution (1 mL per step) under gentle agitation. Precipitated anti–CAP antibody was tested for loss of reactivity after precipitation. No significant loss of reactivity was found.

Aliquots (13×1.5 mL) of precipitated anti–rabbit antibody (concentration 1 mg/mL) and 12×1.5 mL aliquots of precipitated anti–CAP antibody (dilution 1:100) were shipped to Turkey, as well as 10 mL of biotinylated CAP succinate. Annex I to this chapter contains provisional assay protocols for ^{125}I solid–phase RIA development, which were transferred to Turkey in addition to the reagents.

2.4. SECOND SYNTHESIS AND CLEAN–UP OF BIOTINYLATED CAP SUCCINATE (2006)

Following the procedure described above, 68 mg CAP succinate sodium salt (Sigma C3787) and 18 mg N–hydroxysuccinimide (Aldrich 13 067–2) were dissolved in 2 mL dimethylformamide (DMF, Merck 8.22275.1) and incubated with 31 mg N,N'–dicyclohexylcarbodiimide (Aldrich D 8000–2) in 2 mL DMF over night at room temperature under gentle agitation. Then 5–(biotinamido) pentylamine (50 mg, Pierce 21345) dissolved in

1.7 mL purified H_2O was added to the above reaction product and incubated for 4 h under gentle agitation (precipitate formed). The product was centrifuged for 10 min. The supernatant was taken and diluted to a final volume of 50 mL with MeOH/H_2O (50/50, v/v).

The product of synthesis was unstable and required further clean–up to get a stable product. Hence, we applied 25 mL diluted supernatant onto a RP–18 LC column (110626 Lobar® 440–37 LiChroprep® RP–18 (40 µm–63 µm), 440×37 mm; Merck, Darmstadt, Germany) for clean–up using MeOH:H_2O (50:50, v/v) as mobile phase. After discarding the first 100 mL of mobile phase, 2 mL to 3 mL fractions were collected (retention time 3 min) and each second fraction tested in an enzyme immunoassay protocol with streptavidin–HRP as ^{125}I–labelled streptavidin surrogate for the suitable biotinylated–CAP product. Fractions were diluted 1: 200 with assay buffer (PBS pH 7.2) and tested for simultaneous binding to anti–CAP antiserum and streptavidin–HRP: 5 µL (1:200) diluted fractions and 100 µL of anti–CAP antiserum (code R684; 1:150,000; supplied by QUB, Belfast) were incubated over night at 4°C in anti–rabbit IgG coated microtitre plates (1 µg IgG/well; blocked with BSA).

2.5. TRANSFER OF KEY REAGENTS TO THE BRAZILIAN PROJECT PARTNER

The first (anti–rabbit) and second (anti–CAP) antibody were precipitated with ammonia sulphate following a standard operation protocol of the Institute of Physiology. Briefly, 158.2 g ammonia sulphate were added to 187 mL sheep–anti rabbit IgG solution (2 mg/mL) step by step. After precipitation of protein, H_2O (374 mL) was added. Bovine serum albumin (200 mg) was dissolved in 4.8 mL assay buffer (40mM NaH_2PO_4/Na_2HPO_4, 150mM NaCl, pH 7.2, 0.1% bovine serum albumin) and antiserum R684 (0.2 mL) was added. Protein was precipitated stepwise by adding 15 mL of saturated ammonia sulphate solution (1 mL per step) under gentle agitation. Precipitated anti–CAP antibody was tested for loss of reactivity after precipitation. No significant loss of reactivity was found. Lyophilized biotinylated–CAP and second antibody both tested by CAP EIA were sent to Brazil to test in an RIA format.

3. RESULTS AND DISCUSSION

3.1. FIRST SYNTHESIS AND CLEAN–UP (2002/2003)

The chromatographic retention time of CAP succinate determined at the absorption maximum 278 nm was 6.3 min. After chromatographic separation of the synthesis mixture a new peak at 14.5 min emerged. The synthesized product appeared to be biotinylated CAP succinate, due to its UV absorption at 278 nm and binding both to anti–CAP antibody and streptavidin–HRP. The sensitivity of the tested standard curves showed that the tracer was fit for purpose.

On the antibody titration, the fraction collected at 14.5–15 min gave the most intensive signal (i.e binding both to anti–CAP antibody and streptavidin–HRP). The fraction at 14.5–15 min therefore contained the highest concentration of biotinylated CAP succinate. The CAP standard curve assayed (antibody dilution 1:160,000; biotinylated CAP succinate 1:100, 000; streptavidin HRP 1:10,000) according to the described protocol with fraction "14.5–15 min" as tracer showed that the sensitivity was good, with 50% relative binding at 6 pg/well.

The retention time of biotinylated CAP succinate and the sensitivity of the enzyme immunoassay were reproducible with fractions collected later on during the large–scale purification phase. CAP calibration curves (inverse sigmoids) were constructed with selected HPLC fractions of different separations (injection 11 and 21), quality check and proof of reproducibility of HPLC clean–up procedure.

3.2. SECOND SYNTHESIS AND CLEAN–UP (2002/2003)

Fractions 365 to 385 showed high titre. Therefore fraction 365 and 385 were tested with a CAP calibration curve according to the standard enzyme immunoassay protocol [4] at fraction dilution 1:300,000 and antibody dilution 1:150,000 (based on titre test). Typical calibration curves obtained were sigmoid. It can be depicted that both fractions give a typical calibration curve with comparable sensitivity.

Fractions (365 to 385) were pooled to get a final volume of 60 mL. Aliquots (1 mL) were prepared and lyophilized for easy storage and transport. Lyophilized aliquots were further reconstituted in 1 mL assay buffer and tested for activity in the standard CAP enzyme immunoassay. Calibration curves before and after lyophilization, were more linear. To guarantee economical consumption of the valuable antiserum and best possible sensitivity double–antibody technique was chosen. Here tubes are precoated with anti-rabbit antibody, diluted anti-CAP antibody from rabbit is added during the test incubation.

125Iodine–125 labelled CAP is not commercially available. Direct labelling of CAP is rather difficult and labour–intensive for a standard surveillance laboratory for which this test should be fit. As ^{125}I–labelled streptavidin is commercially available, we thought of making use of the biotin–streptavidin system. D–biotin is a naturally occurring growth factor present in small amounts within every cell. It is a cofactor and transporter of CO_2 in numerous processes involving carboxylation reactions. Streptavidin from *Streptomyces avidinii* (MW 60 kD) is a protein consisting of four subunits, each with a single biotin binding site. The biotin–streptavidin interaction is one of the strongest non–covalent affinities known ($K_a = 10^{15}$ M^{-1}). Several enzyme immunoassay procedures using the biotin–streptavidin interaction are described in literature [3]. Analytes of interest are biotinylated and streptavidin–labelled HRP is used for detection. As ^{125}I–labelled streptavidin is also commercially available or it can be more easily synthesized than most ^{125}I–labelled drugs of interest, transfer of this technique to radioimmunoassays was our method of choice.

For both ELISA and RIA, the biotin–streptavidin system has the advantage of one single label fit for several assays. HRP–labels as well as ^{125}I labels have a short shelf–life making regular labelling or acquisition necessary. If the label can be used for assaying several analytes, there will be economical benefits, because one can use most of the labels before the end of their shelf life is reached, which is often not possible in labs with small sample throughput.

As there was no experience with iodine labelling of proteins at the laboratory of the Turkish research contract holder, in the first phase of the project it was planned to acquire commercially produced ^{125}I–labelled streptavidin. At a later stage of the project, in–houselabelling of streptavidin was planned, which should be easily feasible with a procedure based on standard protocols for iodine labelling of proteins.

For optimal assay design biotinylation of CAP had to take into account the structure of the antigen used for specific antibody production. The specific antibody was produced against CAP–glucuronide. As CAP–glucuronide is not commercially available we searched for another CAP derivative useful for labelling. Use of an alternative with more suitable bridge heterology might also give the benefit of better sensitivity. Our enquiries led us to CAP–succinate as surrogate. It bears the CAP moiety plus a small spacer with an easily accessible functional group for coupling.

Possible biotinylation reagents for carboxylic acid groups are biotinylhydrazide, biotinyllysinethylenediamine and 5–(biotinamido) pentylamine (biotinylcadaverine). The latter was chosen for a stable peptide synthesis via the carbodiimide method, as it offers the benefit of a five methylene–spacer arm. The reaction scheme is as follows: N, N'–dicyclohexylcarbodiimide reacts with the carboxyl function of CAP succinate and forms an amine–reactive intermediate, an O–acylisourea. This intermediate is unstable. Therefore N–hydroxysuccinimide is added for stabilization. In a second step the activated product reacts with the amine function of 5–(biotinamido) pentylamine and forms a stable peptide bond (carbodiimid reaction).

4. CONCLUSION

A new concept of ^{125}I solid–phase RIA was developed. It is applicable to a broad array of analytes. After adoption a lab may need only one radioactive label for all tests. This makes analytical procedures much easier. Coupling of 5–(biotinamido)–pentylamine to the carboxylic groups of haptens proved to be an effective means of biotinylation, and this should also be applicable to stilbene or steroid glucuronides. The first results invoving the specific antiserum (in combination with biotinylated CAP succinate and streptavidin HRP) were promising. Thus the ^{125}I–streptavidin solid–phase RIA concept should be pursued further.

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ANNEX I.

^{125}I SOLID–PHASE RIA FOR CAP

I-1. COATING AND ASSAY PROCEDURES

Two dimensional titration of tracer and antibody–provisional protocols sent to research contract holders in Turkey and Brazil.

I-1.1. Reagents and buffers

I-1.1.1. 1st antibody (anti–rabbit from sheep; Weihenstephan)

Stock solution in 75% $(NH_4)_2SO_4$ containing 1 mg/mL (store at 4°C; do not freeze!)

I-1.1.2. 2nd antibody (anti–CAP from rabbit; Queens University Belfast)

Antiserum Code R684 in 75% $(NH_4)_2SO_4$ (dilution 1:100) (store at 4°C; do not freeze!)

I-1.1.3. Biotinylated CAP (Weihenstephan)

Stock solution in purified (deionized/0.22 µm membrane–filtrated) H_2O

Prepare small aliquots in glass vials and store at -20°C.

Take 20 µL stock solution and dilute with 1,980 µL assay buffer (1:100 stock dilution). Prepare aliquots of 100 µL and store at -20°C.

I-1.1.4. ^{125}I–CAP

Amersham code number IM 236–50 µCi in 500 µL

Dilute 100 µL with 9,900 µL assay buffer (stock solution 1:100; that is 500 nCi/500 µL; 1 nCi = 2.2×10^3 dpm). Store at 4°C.

I-1.1.5. Coating buffer

Place about 70 mL distilled/de–ionized H_2O in a 100 mL volumetric flask, add one carbonate/bicarbonate tablet and shake until it is completely dissolved (may take up to 15 min). Add distilled H_2O to make up to the 100 mL mark (this is 0.05M carbonate buffer with a pH 9.6 ± 0.05).

I-1.1.6. Diluent buffer (PBS)

Place about 500 mL distilled/deionized H_2O in a beaker, add one PBS tablet (0.14M NaCl, 3 mM KCl) and stir until completely dissolved. Transfer the solution to a 1 L volumetric flask and add distilled H_2O to make up to the mark (this is 0.01M phosphate buffered saline with a pH 7.4 ± 0.2).

I-1.1.7. Assay buffer (PBS + BSA)

Weigh out 1 g BSA (bovine serum albumin) and transfer into a glass beaker with 1,000 mL diluent buffer (PBS). Wait about 15 min until the protein is dissolved, then mix using a clean glass rod.

I-1.1.8. Washing solution

Tween 80, 0.05% (Polyoxyethylensorbitanmonooleate; Merck Nr. 822187) in distilled/ deionized H_2O

I-1.2. Day 1 coating

I-1.2.1. Prepare 1st antibody coating solution

Transfer 250 µL of the antibody stock solution into a 50 mL volumetric flask. Fill the flask to the mark with 0.05M carbonate buffer. This gives an antibody coating solution of 0.5 µg/100 µL; 50 mL is sufficient for coating about 240 tubes.

To ensure that your volumetric flask is free of protein**,** rinse thoroughly with sodium hydroxide solution after use. Afterwards rinse thoroughly with plenty of distilled/deionized H_2O and let it dry.

I-1.2.2. Coat tubes

Dispense 200 µL of anti–rabbit antibody (that is 1 µg) coating solution into each Nunc "star" tube, except the TC tubes. Cover the tubes with parafilm or similar sealing material and incubate overnight (at least 12 h) at 4ºC (in the refrigerator).

To ensure that the antibody solution is pipetted to the bottom of the tube put the tubes into a centrifuge (1,000g, 2 min) after pipetting the antibody.

I-1.3. Day 2 coating

I-1.3.1. Decant tubes:

After incubation, decant the contents of the tubes and tap the mouth of the tubes vigorously on absorbent paper to remove the remaining liquid.

I-1.3.2. Coat tubes:

Dispense 300 µL of assay buffer into each tube, except the TC tubes. Cover the tubes with parafilm or similar sealing material and incubate at room temperature for 30 min.

To ensure that the blocking solution is pipetted to the bottom of the tube put the tubes into a centrifuge (1,000g, 2 min) before incubation.

I-1.3.3. Decant tubes:

After incubation, decant the contents of the tubes. Some liquid may remain in the tube to protect the antibody during freezing.

Cover the tubes with parafilm or similar sealing material. You can store them for at least six months at -20°C in the freezer (Do not use later if defrosted and not used at the time).

I-1.4. Day 1 assay (procedure 1)

I-1.4.1. Label assay tubes

Take the necessary number of coated tubes out of the freezer, defrost for about 5 min and add 500 µL of washing solution to each tube and decant to remove liquid. Rinse the tubes a second time with 500 µL of washing solution, decant, and tap the mouth of the tubes vigorously or absorbent paper to remove the remaining liquid.

Label Nunc "star" tubes in duplicate for total counts (TC, uncoated tubes), NSB non–specific binding, B zero (B_0)

I-1.4.2. Prepare radioactive tracer working solution

Put 10 µL of 1:100 diluted stock solution in a TC tube and count the radioactivity (approx. 20,000 cpm). For 100 tubes dilute 500 µL stock solution (1:10) with 4,500 µL assay buffer (working solution 1:10,000) (TC ~ 10,000 cpm per 50 µL).

I-1.4.3. Set up assay

Allow all components to reach room temperature (~10 min) before setting up the assay. Gently mix or lightly vortex all reagents (antibody, ^{125}I tracer, biotinylated CAP–succinate) before pipetting. Dilute them in assay buffer according to the protocol below. Take 50 µL diluted biotinylated CAP–succinate, 50 µL diluted antibody and 50 µL diluted tracer for the assay as described below.

I-1.4.3.1. Biotinylated CAP–succinate:

- 1:2,500 (80 µL stock dilution 1:100 + 1,920 µL assay buffer)
- 1:5,000 (1,000 µL assay buffer + 1,000 µL stock dilution 1:2,500),
- 1:10,000 (1,000 µL assay buffer + 1,000 µL stock dilution 1:5,000),
- 1:20,000 (1,000 µL assay buffer + 1,000 µL stock dilution 1:10,000)

I-1.4.3.2. Antibody:

- 1:10,000 (20 µL stock dilution 1:100 + 1,980 µL assay buffer),
- 1:20,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:10,000),
- 1:40,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:20,000),
- 1:80,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:40,000),
- 1:160,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:80,000),
- 1:320,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:160,000),
- 1:640,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:320,000)

I-1.4.3.3. Pipetting scheme (in duplicate):

- NSB 0: coated tube, 100 µL assay buffer

- NSB 1:2,500: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL assay buffer
- NSB 1:5,000: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL assay buffer
- NSB 1:10,000: coated tube, 50 µL 1: 10,000 diluted biotinylated CAP–succinate, 50 µL assay buffer
- NSB 1:20,000: coated tube, 50 µL 1: 20,000 diluted biotinylated CAP–succinate, 50 µL assay buffer
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:40,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:160,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:320 000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:40 000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:5000 diluted biotinylated CAP–succinate, 50 µL antibody 1:160 000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:320,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:10 000 diluted biotinylated CAP–succinate, 50 µL antibody 1:40 000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:160,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:320,000

- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µLantibody 1:20,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:40,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:160,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:320,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000
- TC: uncoated tube, 50 µL ^{125}I–tracer
- Finally add 50 µL ^{125}I–tracer to all tubes (NSB tubes and B0 tubes)

After pipetting lightly vortex the mixture, put the tubes into a centrifuge (1,000g, 2 min), cover with parafilm and incubate overnight (at least 12 h) at 4ºC.

I-1.5. (D) – Day 2 assay (procedure 1)

I-1.5.1. Washing:

After incubation, remove the TC tubes from the rack and vigorously decant all the remaining tubes into an appropriate radioactive waste disposal container and allow them to drain for five minutes on absorbent paper.

Add 500 µL of washing solution to each tube (except TC) and decant to remove liquid as described above.

Rinse the tubes a second time with 500 µL of washing solution and decant as described above.

I-1.5.2. Measure radioactivity and calculate results:

Count the radioactivity of each tube (including TC) in a gamma counter for a fixed time (normally 60 sec).

I-1.6. (C) – Day 1 assay (procedure 2)

I-1.6.1. Label assay tubes:

Take the necessary number of coated tubes out of the freezer, defrost for about 5 min and add 500 µL of washing solution to each tube and decant to remove liquid. Rinse the tubes a

second time with 500 µL of washing solution, decant, and tap the mouth of the tubes vigorously or absorbent paper to remove the remaining liquid.

Label Nunc "star" tubes in duplicate for total counts (TC, uncoated tubes), NSB non–specific binding, B zero (B_0)

I-1.6.2. Set up assay:

Allow all components to reach room temperature (~10 min) before setting up the assay. Gently mix or lightly vortex all reagents (antibody ^{125}I tracer, biotinylated CAP–succinate) before pipetting. Dilute them in assay buffer according to the protocol below. Take 50 µL diluted biotinylated CAP–succinate, 50 µL diluted antibody and 50 µL diluted tracer for the assay as described below.

I-1.6.2.1. Biotinylated CAP–succinate:

- 1:2,500 (80 µL stock dilution 1:100 + 1,920 µL assay buffer)
- 1:5,000 (1,000 µL assay buffer + 1,000 µL stock dilution 1:2,500),
- 1:10,000 (1,000 µL assay buffer + 1,000 µL stock dilution 1: 5,000),
- 1:20,000 (1,000 µL assay buffer + 1,000 µL stock dilution 1:10,000)

I-1.6.2.2. Antibody:

- 1:10,000 (20 µL stock dilution 1:100 + 1980 µL assay buffer),
- 1:20,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:10,000),
- 1:40,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:20,000),
- 1:80,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:40,000),
- 1:160,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:80,000),
- 1:320,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:160,000),
- 1:640,000 (1,000 µL assay buffer + 1,000 µL antibody dilution 1:320,000)

I-1.6.2.3. Pipetting scheme (in duplicate):

- NSB 0: coated tube, 100 µL assay buffer
- NSB 1:2,500: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL assay buffer
- NSB 1:5,000: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL assay buffer
- NSB 1:10,000: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL assay buffer
- NSB 1:20,000: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL assay buffer
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:40,000

- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:160,000
- B_0: coated tube, 50 µL 1:2,500 diluted biotinylated CAP–succinate, 50 µL antibody 1:320,000
- B_0: coated tube, 50 µL 1:2500 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:40,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:80 000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:160,000
- B_0: coated tube, 50 µL 1:5,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:320,000
- B_0: coated tube, 50 µL 1:5000 diluted biotinylated CAP–succinate, 50 µL antibody 1:640 000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:40,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:160 000
- B_0: coated tube, 50 µL 1:10 000 diluted biotinylated CAP–succinate, 50 µL antibody 1:320 000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:10,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µLantibody 1:10,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:20,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:40,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:80,000
- B_0: coated tube, 50 µL 1:20,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:160,000

- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:320,000
- B_0: coated tube, 50 µL 1:10,000 diluted biotinylated CAP–succinate, 50 µL antibody 1:640,000

After pipetting, lightly vortex the mixture, put the tubes into a centrifuge (1,000g, 2 min), cover with parafilm and incubate overnight (at least 12 h) at 4ºC.

I-1.7. (D) – Day 2 assay (procedure 2)

I-1.7.1. Prepare radioactive tracer working solution:

Put 10 µL of 1:100 diluted stock solution in a TC tube and count the radioactivity (approx. 20,000 cpm). For 100 tubes dilute 500 µL stock solution (1:10) with 4,500 µL assay buffer (working solution 1:10,000) (TC ~ 10,000 cpm per 50 µL).

I-1.7.2. Washing:

After incubation, remove the TC tubes from the rack and vigorously decant all the remaining tubes into an appropriate radioactive waste disposal container and allow them to drain for five minutes on absorbent paper.

Add 500 µL of washing solution to each tube (except TC) and decant to remove liquid as described above.

Rinse the tubes a second time with 500 µL of washing solution and decant as described above.

I-1.7.3. Add Tracer

- TC: uncoated tube, 50 µL ^{125}I tracer
- Add 50 µL ^{125}I tracer to all tubes (NSB tubes and B_0 tubes)

I-1.7.4. Washing:

After incubation, remove the TC tubes from the rack and vigorously decant all the remaining tubes into an appropriate radioactive waste disposal container and allow them to drain for five minutes on absorbent paper.

Add 500 µL of washing solution to each tube (except TC) and decant to remove liquid as described above.

Rinse the tubes a second time with 500 µL of washing solution and decant as described above.

I-1.7.5. Measure radioactivity and calculate results:

Count the radioactivity of each tube (including TC) in a gamma counter for a fixed time (normally 60 sec).

After pipetting lightly vortex the mixture, put the tubes into a centrifuge (1,000g, 2 min), cover with parafilm and incubate overnight (at least 12 h) at 4ºC.

DETERMINATION OF BENZIMIDAZOLES AND AVERMECTIN RESIDUES IN BOVINE MILK BY LIQUID CHROMATOGRAPHY WITH TANDEM MASS SPECTROMETRY AND FLUORESCENCE DETECTION

LUCAS, O
National Agricultural Health Service, SENASA, Perú

Abstract

Liquid chromatography tandem mass spectrometry (LC–MS/MS) and high performance chromatography fluorescence detector (HPLC–FLD) methods were developed and applied to test ten benzimidazoles and avermectin anthelmintic residues: Albendazole (ABZ), Albendazole sulphoxide (ABZ–SO), Albendazole sulphone (ABZ–SO2), Thiabendazole (TBZ), Triclabendazole (TCBZ), Triclabendazole–sulphoxide (TCBZ–SO), Triclabendazole-sulphone (TCBZ–SO_2), Abamectin (ABA), Emamectin (EMA) and Ivermectin (IVE) in milk for human consumption. This includes a simple modification of the QuEChERS method for the extraction of the analytes from milk. Analytes were extracted with Acetonitrile in presence of $MgSO_4$ and NaCl (for induction of phase separation), followed by dispersive solid–phase clean up with a mixture of magnesium sulphate and C_{18}. Results show good selectivity and the confirmatory analysis met the EU tolerances. Two transition ions were monitored for the confirmatory method. The majority of analytes met EU validation requirements with recovery values typically in the range 80%–110% in milk. Analysis of the avermectins by LC–FLD met EU validation requirements with recovery values typically in the range 80%–110%. The majority of the analytes also gave < 14% RSD at each level of fortification. On the basis of the results obtained in this research, it can be concluded that the analytical method for benzimidazol and avermectin residues in milk by LC–MS/MS and FLD comply with EU recommendation for analytical method for veterinary drugs residue analysis and can be used for routine analyses in official monitoring program.

1. INTRODUCTION

Benzimidazoles and avermectins are the most frequently used anthelmintics in animal husbandry for protecting against or treating gastrointestinal nematodes, lungworms and liver fluke [1, 2]. However, if the recommended withdrawal times are not respected or if dosages are higher than recommended, the residue levels in animal products, such as bovine milk, muscle and other edible tissue, can be higher than the maximum residue limit (MRL). Anthelmintic residues in food cause some toxic effect in consumers, including teratogenic and embryotoxic effects. Moreover, some benzimidazoles, metabolites are more toxic than the parent drug. The Codex Alimentarius and the national or regional food safety authorities have set MRLs to protect consumer health. For example Codex [3] and EU [4] MRLs for benzimidazoles and avermectins in bovine muscle and milk range from 10 µg/kg to 225 µg/kg.

The EU legislation does not authorize use of triclabendazole, mebendazole, doramectin and abamectin in animals that produce milk for human consumption. Marker residues of benzimidazoles include mainly benzimidazole metabolites and specific compound for avermectins, resulting in a total of 21 target compounds that should be analysed.

Benzimidazole and avermectin anthelmintics residues have mostly been determined by liquid chromatography with UV or FLDs [1, 2]. However, these methods require extensive clean–up, detect a limited number of compounds and often require derivatization of analytes. The LC–MS/MS technique on the other hand is more sensitive and specific than HPLC with UV or FLD [5]. Moreover, MS techniques also enable simultaneous quantitative determination of several compounds (multi–residue method) with the same chromatographic system and extraction process [6]. The high selectivity of LC–MS/MS may require relatively simple and

fast sample extraction processes and is a good analytical tool for routine regulatory laboratories.

De Ruyck et al. [7] reported a validated analytical method for residues of benzimidazoles in milk, involving extraction with ethyl acetate followed by LC separation and detection with LC–MS/MS with electrospray ionization (ESI). The main advantage of this method is the relatively simple and fast sample extraction process that. Nevertheless, this method was validated only for bovine milk samples. Due to the increased consumption of bovine muscle locally and internationally, it was important to expand and validate the above–mentioned fast and simple analytical method for simultaneous determination of benzimidazoles and avermectins residues in bovine muscle in addition to milk.

The aim of this research was therefore to develop and validate an analytical method for simultaneous determination of benzimidazole and avermectin anthelmintic residues in bovine muscle and milk by LC–MS/MS, in order to provide to official national laboratories a fast and relatively simple validated analytical method for their monitoring program.

2. MATERIALS AND METHODS

2.1. LC–MS/MS

A simple modification of the QuEChERS method for the extraction of the analytes from milk samples was used. This method includes the extraction of the analytes with acetonitrile (MeCN) in presence of $MgSO_4$ and NaCl for phase separation, followed by dispersive solid–phase extraction with a mixture of magnesium sulphate and C18.

2.1.1. Optimization of chromatographic parameters

During the optimization of the chromatographic conditions two different mobile phases was tested: (A) 0.1% aqueous formic acid and (B) MeCN and 2) (A) Ammonium formate 25mM aqueous and (B) Ammonium formate 12.5mM in MeOH:MeCN (50:50, v/v). In both cases Gradient elution (0.3 mL/min) with an initial starting condition of 98:2 (A:B, v/v), was used, and the mobile phase B increased to 100% over 5 min, and held for 10.5 min before it returned to the initial starting condition.

The LC system was equilibrated for at least 30 min with mobile phase A:B (2%:98%), at the injection volume of 10 μL. With mobile phase 2 higher chromatographic signals were seen with mobile phase 2 than with mobile phase 1, probably because ammonium formate enhances the positive ESI process.

2.1.2. MS conditions

The MS conditions were optimized by infusion of a 1 μg/mL standard solution of each analyte and monitoring the two most abundant fragment ions produced from the molecular ion. The ionization parameters were: ESI Positive, source temperature: 120^{o}C, desolvation temperature: 350^{o}C, cone gas: 50 (L/h) and desolvation gas: 800 L/h. The results are summarized in Table 1.

TABLE 1. MS RESULTS

#	Analyte	MS/MS Transition Base peak (1) Second peak (2)		Cone Voltage (V)	Collision Energy (eV)	Dwell Time (sec)	Function
1	TBZ	202.1 > 175.1	(1)	50	30	0.20	I
		202.1 > 131.1	(2)	50	28		
2	ABZ	266.2 > 234.2	(1)	40	15	0.20	I
		266.2 > 191.1		40	30		
3	ABZ–SO_2	298.1 > 265.9	(2)	40	28	0.20	I
		298.1 > 159.1		40	15		
4	ABZ–SO	282.1 > 239.9	(1)	40	28	0.20	I
		282.1 > 208		40	15		
5	TCBZ	359 > 274.1	(2)	50	25	0.20	II
		359 > 344.1		50	28		
6	TCBZ–SO_2	391 > 242.3	(1)	50	40	0.10	II
7	TCBZ–SO	375 > 242.2	(2)	30	45	0.10	II
8	EMA	886.5 > 158.1	(1)	50	30	0.10	I
		886.5 > 126		50	15	0.10	
9	ABA	891 > 305.1	(2)	50	30	0.10	II
		891 > 567.2		50	12	0.10	
10	IVE	892.5 > 569.2	(1)	50	30	0.10	II
		892.5 > 307.1		50	12	0.10	
11	Triphenyl phoshate	327 > 77	(2)	25	20	0.20	II
12	Cyprodinil	226 > 108	(1)	40	25	0.20	II

2.1.3. Method validation

The validation procedure was carried out in accordance to EU legislation and included the following parameters: recovery, repeatability, selectivity, linearity, CCα and CCβ. The repeatability and recovery parameters were determined using six blank milk samples spiked with a mixed solution of the 10 analytes at level 50 µg/kg, 100 µg/kg and 150 µg/kg, equivalent to 0.5, 1.0 and 1.5 times the MRLs, respectively. Six independent replicate analyses were carried out for each level and the experiments were repeated on two additional occasions (for a total of 18 analyses per level). Blank milk samples for the validation experiments were obtained from commercial market and analysed to rule out prior contamination with the target analytes.

Selectivity was determined by analysing blank and spiked blank samples to demonstrate the absence of chromatographic interferences. This was in addition to LC–MS/MS confirmatory tests analysis where two MSMS transitions for each analyte were determined according to EC 2002/657 [8]. Relative ion intensities (from standard and analytical samples used for the validation, at comparable concentrations) were measured under the same conditions.

The CCα and CCβ were determined using blank milk samples spiked with the analytes at around the permitted limit in equidistant steps (50 ng/g, 100 ng/g and 150 ng/g) and after identification, signals were plotted against the added concentration. The corresponding concentration at the permitted limit plus 1.64 times the standard deviation of the within–laboratory reproducibility equalled the decision limit (α=5%), while and the corresponding concentration at the value of the decision limit plus 1.64 times the standard deviation of the within–laboratory reproducibility equals the detection capability (β=5%).

2.2. THE HPLC–FLD

This method involved extraction of samples with organic solvents and clean–up by solid phase extraction (SPE) followed by derivatization of analytes in order to form fluorescent compounds that are separated and quantified using HPLC with FLD. The analytical procedure is showed in the following flowchart and the chromatographic parameters in Table 2. Samples were extracted in acetonitrile and water and cleaned using C18 SPE material in the presence of N–acetylimidazole and trifluoroacetic acid anhydride before by HPLC–FLD.

TABLE 2. CHROMATOGRAPHIC CONDITIONS

Analytical column	Phase reverse C18, 4.6×150 mm, 3.5 µm
Mobile phase	MeOH:H_2O (97:3, v/v).
Condition of flow	Isocratic
Flow rate	1 mL/min
Injection volume	20 µL
Temperature (sample)	25°C
Temperature (column)	30°C
Run time	9 min
Detector	FLD 2475
Excitation wavelength	365 nm
Emission wavelength	465 nm

3. RESULTS AND DISCUSSION

3.1. LC–MS/MS

The majority of analytes met EU validation requirements with recovery values typically in the range 80%–110% in milk. However, some analytes, mainly the avermectins, did not meet this requirement. Most analytes also had RSDs of <14% at each fortification level. One possible explanation could be that the extraction procedure was not suitable enough for simultaneously isolation of benzimidazoles and avermectins. It was therefore necessary to investigate the use of LC–FLD for avermectins; the LC–MS/MS was maintained as the method of choice for the benzimidazoles [1, 2].

Linearity was demonstrated for all 10 analytes by preparing a five–point matrix-matched calibration curve at concentration levels of 0 µg/kg, 5 µg/kg, 100 µg/kg, 250 µg/kg and 500 µg/kg. Most analytes had linear regression values greater than 0.99.

The CCα and CCβ values for benzimidazoles (Table 3) were calculated at MRL level (100 µg/kg) using matrix matched calibration curve (50 µg/kg, 100 µg/kg and 150 µg/kg) (n=6 replicates by level). The CCα and CCβ values for avermectins were not determined. Therefore, it was necessary to carry out additional experiments assuming an MRL value of 10 µg/kg for the three avermectins in milk, including accuracy and precision (Table 4).

TABLE 3. ANALYTICAL LIMITS FOR SEVEN BENZIMIDAZOLES

	CCα (µg/kg)	CCβ (µg/kg)	MRL (µg/kg)
TCBZ	128	156	100*
TCBZ–SO_2	139	177	100
TCBZ–SO	139	178	100
TBZ	146	193	100
ABZ	137.0	174.0	100
ABZ–SO_2	155	210	100
ABZ–SO	160	219.0	100

* MRL for other tissue, there is not MRL for TCB in milk

TABLE 4. RECOVERY AND REPEATABILITY OBTAINED FROM THE METHOD VALIDATION EXPERIMENTS

Analyte	Fortification Level (µg/kg)	Recovery % (n = 6 replicates by level)								Acceptable Range *	
		Day 1		Day 2		Day 3		REC	REP	REC	REP
		Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD	Mean	RSD
TCBZ	50.0	96.4	7.4	81.7	10.6	95.6	5.0	91.2	9.0	80-110	<17
	100	82.5	6.7	77.3	4.9	86.9	3.4	82.2	5.9		<15
	150	78.8	5.9	69.1	5.4	88.1	18.4	78.7	12.1		<14
TCBZ–SO_2	50.0	96.1	8.6	88.2	11.1	92.2	5.5	92.2	4.2	80-110	<17
	100	91.3	8.8	75.4	3.9	91.3	3.7	86.0	10.7		<15
	150	89.2	5.2	69.4	5.0	110	19.9	89.6	22.9		<14
TCBZ–SO	50.0	85.9	8.5	68.5	12.8	83.5	4.3	79.3	11.9	80-110	<17
	100	73.9	7.8	64.1	5.6	79.4	4.3	72.5	10.7		<15
	150	74.4	4.5	59.7	6.1	91.6	22.3	75.2	21.3		<14
TBZ	50.0	88.9	7.4	62.8	16.4	64.6	4.2	72.1	20.2	80-110	<17
	100	78.5	5.9	56.2	11.8	69.6	6.3	68.1	16.5		<15
	150	79.2	5.0	50.2	4.4	82.5	26.9	70.6	25.2		<14
ABZ	50.0	105.0	6.7	94.2	12.2	108	4.3	102.3	7.0	80-110	<17
	100	86.3	7.5	88.0	4.7	100	4.2	91.5	8.3		<15
	150	83.3	7.2	80.3	4.5	106	25.6	89.9	15.7		<14
ABZ–SO_2	50.0	100.2	8.0	42.9	26.4	80.5	8.2	74.5	39.1	80-110	<17
	100	67.3	10.1	62.9	4.2	76.9	6.9	69.0	10.3		<15
	150	66.7	9.5	72.3	6.6	60.9	37.3	66.6	8.6		<14
ABZ–SO	50.0	115.3	7.7	67.2	14.8	115	6.0	99.0	27.8	80-110	<17
	100	80.7	8.9	80.6	4.4	97.0	5.4	86.1	11.0		<15
	150	66.7	7.5	72.3	4.9	60.9	36.8	66.6	8.6		<14
ABA	50.0	84.8	10.6	78.3	10.5	92.7	8.1	85.3	8.5	80-110	<17
	100	65.0	5.2	75.0	6.1	46.4	8.1	62.1	23.4		<15
	150	68.7	12.4	67.2	13.4	66.5	20.4	67.5	1.7		<14
EMA	50.0	78.4	4.6	62.5	6.3	70.3	3.6	70.4	11.3	80-110	<17
	100	66.1	8.8	57.6	3.9	69.0	4.1	64.2	9.3		<15
	150	65.0	8.3	52.5	4.1	70.0	27.7	62.5	14.4		<14
IVER	50.0	74.3	13.3	82.4	19.5	86.7	17.0	81.1	7.8	80-110	<17
	100	71.3	13.5	62.9	12.0	78.6	5.1	70.9	11.1		<15
	150	74.4	6.0	58.9	6.9	83.8	28.6	72.3	17.4		<14

*[8]

3.2. HPLC–FLD

3.2.1. Chromatographic conditions

Good chromatographic separation of the 5 avermectins EPRI (3.83 min) EMA (5.04 min), ABA (5.8 min), DOR (6.9 min) and IVER (7.9 min) was obtained. Instability of the standards had an impact on the chromatography since poor eprinomectin separation was noted with a

standard suspected to have degraded, unlike when a different (fresher) analytical standard was used.

3.2.2. Results of the method validation

The linear regression values for most analytes were greater than 0.99: DOR, 0.9993; EMA, 0.992; and 0.999 for IVER and ABA. Good precision and recovery values were detected (Table 5).

TABLE 5. ACCURACY AND PRECISION TEST RESULTS

ABAMECTIN

Analyst	Day	Analysis	Fortification level 1 (ng/g) 2.5		Fortification level 2 (ng/g) 5		Fortification level 3 (ng/g) 10	
			CE[a]	% R[c]	CE	% R	CE	% R
FC	1	1	2.876	115	2.647	53	7.461	75
		2	1.994	79.76	3.772	75	8.489	85
	15/08/13	3	2.828	113	3.65	73	8.399	84
		4	2.136	85.44	3.652	73	9.428	94
		5	2.197	87.88	3.82	76	8.413	84
% R		Average	96.25		70.16		84.38	
		Desv. est	16.56		9.74		6.96	
		RSD (%)	17.20		13.89		8.25	
Acceptance criteria			RSD (%) <	26.41	RSD (%) <	23.80	RSD (%)	<21.29

DORAMECTIN

Analyst	Day	Analysis	Fortification level 1 (ng/g) 2.5		Fortification level 2 (ng/g) 5		Fortification level 3 (ng/g) 10	
			CE[a]	% R[c]	CE	% R	CE	% R
FC	1	1	2.583	103	4.256	85	7.524	75
		2	2.881	115	4.191	84	8.878	89
	15/08/13	3	2.828	113	4.376	88	8.701	87
		4	2.504	100	4.658	93	8.652	87
		5	2.775	111.00	4.091	82	8.60	86
% R		Average	109		86.29		84.71	
		Desv. est	6.51		4.36		5.40	
		RSD (%)	5.99		5.06		6.37	
Acceptance criteria			RSD (%) <	26.41	RSD (%) <	23.80	RSD (%)	<21.29

EMAMECTIN

Analyst	Day	Analysis	Fortification level 1 (ng/g) 2.5		Fortification level 2 (ng/g) 5		Fortification level 3 (ng/g) 10	
			CE[a]	% R[c]	CE	% R	CE	% R
FC	1	1	2.746	110	5.048	101	8.864	89
		2	2.909	116	4.552	91	8.635	86
	15/08/13	3	2.535	101	5.025	101	8.825	88
		4	2.935	117	4.656	93	7.652	77
		5	2.775	111.00	4.091	82	8.52	85
% R		Average	111.		93.49		84.99	
		Desv. est	6.38		7.86		4.94	
		RSD (%)	5.74		8.41		5.81	
Acceptance criteria			RSD (%) <	26.41	RSD (%) <	23.80	RSD (%)	<21.29

IVERMECTIN								
Analyst	**Day**	**Analysis**	**Fortification level 1 (ng/g) 2.5**		**Fortification level 2 (ng/g) 5**		**Fortification level 3 (ng/g) 10**	
			CE[a]	**% R[c]**	**CE**	**% R**	**CE**	**% R**
FC	1	1	2.691	108	4.792	96	8.906	89
		2	2.537	102	4.569	91	8.459	85
	15/08/13	3	2.674	107	4.231	85	8.177	82
		4	2.333	93.32	4.638	93	8.513	85
		5	2.661	106	4.091	82	8.83	88
% R		Average	103		89.28		85.77	
		Desv. est	6.02		5.85		2.96	
		RSD (%)	5.83		6.55		3.45	
Acceptance criteria			RSD (%) <	26.41	RSD (%) <	23.80	RSD (%)	<21.29

CE = Experimental Concentration; % R = Percentage of Recovery; RSDH (HORWIST) = RSD Calculated according to Horwitz's equation.

Matrix effect was investigated and the result showed that there is not matrix effect for avermectins in milk.

3.2.3. Application of the analytical method in national monitoring program in Peru

The method was used for benzimidazol residue analysis in milk under the Peruvian national contaminant monitoring program through the laboratory "Toxic Residues and Agricultural Product Control Centre" of SENASA. The program is ongoing.

3.2.4. ISO 17025 accreditation of the SENASA laboratory in Peru

One of the most important contributions of this project was the ISO 17025 accreditation of the laboratory of Toxic Residues and Agricultural Products Control that is the official laboratory in Peru responsible to control of food safety.

4. CONCLUSION

An analytical technique based on LC–MS/MS and FLD detection for benzimidazol and avermectin residues in milk that complies with EU recommendations has been developed/validated and is available for routine analyses in official monitoring program.

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SCREENING METHODS FOR BENZIMIDAZOLES IN ANIMAL PRODUCTS

YANG， S., LIU, H., CHEN, G., ZHIHUA, Y.,
Institute of Quality Standards and Testing Technology for Agro–Products, CAAS, China

Abstract

A competitive Enzyme Linked Immunosorbent Assay (ELISA) has been established to screen residues of albendazole (ABZ) and its metabolites in animal foodstuff with the obtained ABZ anti–serum. The inhibiting curve of B/B_o to logarithmic concentrations in 1 μg/L–1000 μg/L range showed that the Inhibitory Concentration (IC_{50}) was 13.80 μg/L. The average recoveries were 85.2%, 79.4%, 85.8% and 81% for liver, pork, mutton and fish tissue spiked samples, respectively. The percent RSDs were 10.8%, 7.6%, 6.2%, 8.8%. The result showed that the method can meet rapid screening requirements in residue survey.

1. INTRODUCTION

The BZ anthelmintics are a group of chemicals widely used in veterinary practices for treating domestic animals and cultured fish infected by different parasites and diseases, such as gastrointestinal nematodes, lungworms and liver fluke [1, 2]. The drugs are mainly metabolized in liver, which is the target organ, but their residues can also be found in meat when withdrawal period is not long enough or if doses applied are higher than recommended. Therefore, application of BZs in farm animals raises the possibility that residues can also be found in food produced for human consumption [3].

Furthermore, a number of approved BZs have been found to be teratogenic and embryotoxic in some species. Resistance to BZs is exists, widespread and appears to be specifically selected in a variety of nematode parasites [4]. For safe food production, in most of the countries including EU, US and China have set maximal residue limits (MRLs) for BZs and their metabolites in animal products. In the EU legislation, the selected tissues for BZs residue control include liver, kidney, muscle and fat, and the MRLs range from 10 μg/kg to 1,000 μg/kg depending on the compounds and the tissue specificity. Thus, BZs and their metabolites MRLs control are listed in their national residues monitoring programs.

Some ELISA kits based on monoclonal or polyclonal antibodies have been developed to screen BZs in various animal matrix [5–7]. However, such methods still encounter some difficulties and therefore have some limitations for use in national residues [8]. Firstly, for BZs with very low MRLs such as 10 μg/kg, the ELISA method sensitivity is not low enough. Secondly, some commercial kits can't simultaneously detect parent BZs and their metabolites. Therefore, the aim of our project was to develop an ELISA for screening BZs to address the above challenges and meet the demands of residue survey in foodstuff.

2. MATERIALS AND METHODS

2.1. SYNTHESIS OF THE ANTIGEN AND ABZ CONJUGATED WITH ENZYME

Since ABZ contains an ester bond, an alkaline hydrolysis method was applied to form carboxyl group, which was then linked to a carrier protein bovine serum albumin/ovalbumin (BSA/OVA) to form an immunogen; ABZ was also then conjugated to enzyme horse radish peroxidase (HRP).

2.2. PREPARATION OF THE ANTISERUM

Following production of the antigens, 8 rabbits were immunized to produce polyclonal antibodies. Jugular vein blood was taken every two weeks to check the titer values and specific binding with albendazole. At the time when antiserum had the highest titer and specificity, the rabbits were killed to obtain antiserum. This was purified by ammonium sulphate fractionation precipitation, and used to prepare the ELISA kit.

2.3. PROCEDURE OF INDIRECT NON-COMPETITIVE ELISA FOR EVALUATING ANTISERUM

Polystyrene plates were coated with 1 μg/mL concentration of each ABZ–CP–OVA in 0.05 mol/L carbonate–bicarbonate buffer (pH 9.6) and incubated overnight at 4ºC. The plate coating solution was removed with phosphate buffered saline Tween 20 [PBST (0.01 mol/L PBS containing 0.05% Tween 20, pH 7.4)] washed three times and unoccupied sites blocked by incubating with 3% skim milk solution in 0.01 mol/L PBS for 1 h at 37ºC.The blocking solution was then decanted and 50 μL/well ALB standard solution (100 ng/mL) were added.

Also 50 μL/well of antiserum (1:2,000 in PBS) were added again and incubated at 37ºC for 45 min. Washing free antiserum in PBST, 100 μL/well of goat anti–rabbit IgG–HRP (diluted 1:4,000 in PBST) was added and incubated at 37ºC for 45 min, then the plates were washed again, followed by addition of 100 μL/well of substrate solution [0.1% 3.3′,5.5′–tetramethylbenzidine (TMB) in 0.05 mol/L. Acetate citric acid buffer, pH 4.2, containing 2% (v/v) dimethyl sulfoxide (DMSO) and 0.005% H_2O_2] was added and the plates incubated for 10 min. The reaction was terminated by adding 2 mol/L sulphuric acid and the absorbance read at 450 nm.

2.4. PROCEDURE OF DIRECT COMPETITIVE ELISA FOR ALBENDAZOLE DETECTION

Antibody coating: 100 μL coating buffer containing capture antibody was added to the microplate well and incubated for 24 h at 4°C. The coating buffer was discarded and the well washed three times before adding 200 μL of blocking buffer. After 12 h of blocking at 4°C, the blocking buffer was discarded and the well. Then the microplate was dried at 37°C for 1 h.

Detection procedure: for ABZ, 50 μL of sample or standard solution was added to the well followed by the adding 50 μL diluted HRP labeled antigen solution. After incubation for 45 min at 37°C, the wells were washed four times and 50 μL substrate solutions A and B, respectively added. After 10 min of incubation at room temperature, 50 μL 2M sulphuric acid was added and measurement made at 450 nm optical density (OD).

2.5. LINEAR RANGE AND SENSITIVITY OF THE ELISA

Serial ablendazole standard solutions of 1,000 μg/L, 500 μg/L, 200 μg/L, 100 μg/L, 10 μg/L, 5 μg/L, 1 μg/L, 0 μg/L were measured by direct competitive ELISA. The inhibiting curves of B/B_0 to logarithmic concentrations were made to get IC_{50}. The sensitivity of the ELISA can be determined by concentration of OD value at 0 μg/L, by 2×SD on calibration curve.

2.6. SAMPLE PREPARATION

To tissues (1g–2 g) or milk (1 mL) in a 50 mL centrifugation tube, 10 mL of PBS containing 10% DMSO was added followed by homogenization for 30 min with ultra–turrax or similar tools. The extract was then centrifuged at 4,500 rpm for 5 min and the clean supernatant transferred to another tube ready for detection.

2.7. VALIDATION OF THE METHOD

2.7.1. Recovery of spiked samples

Pork, porcine liver, mutton and fish were spiked with 200 μg/kg, 100 μg/kg, 10 μg/kg, and 1 μg/kg of ABZ, respectively. Those samples were measured by the ELISA kit with each sample was repeated 6 times. The recoveries and RSD were calculated.

2.7.2. Comparison with other commercial ABZ kits

In Chinese domestic market there is only one commercial ELISA kit for ABZ available named Commercial A. For the two kits, standard curves and cross reaction were measured, and 200 μg/kg, 100 μg/kg, 10 μg/kg, 1 μg/kg of spiked sample for pork, liver, mutton and fish were analysed, respectively.

2.8. CROSS REACTION

Six BZs namely ABZ, ABZ–SO_2, ABZ–SO, Oxibendazole (OXI), Oxfendazole (OFZ) AND Fenbendazole (FBZ), were measured by the developed ELISA kit for the cross reactivity with same procedure as described.

3. RESULTS AND DISCUSSION

3.1.CONJUGATED DRUGS AND CARRIER PROTEINS

On comparison of ABZ before and after synthesis, and free carriers, the UV scanning showed that ABZ conjugated with HRP had a higher absorption at 295 nm than free HRP solution, and an absorption peak at 403 nm compared with free ABZ solution. Thus the results suggest the linkage of ABZ with HRP was a success with a coupling ratio of 3.6:1 (Figure 1).

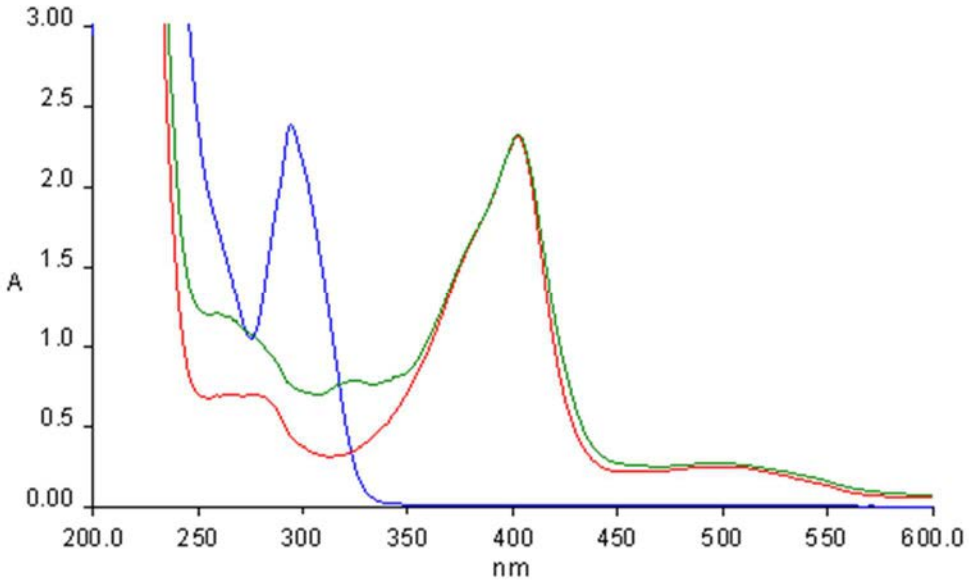

FIG. 1. The ultraviolet absorption spectra of ABZ, HRP–ABZ and HRP, 1: ABZ (100 μg/mL) 2: HRP–ABZ (1 mg/mL) 3: HRP (1 mg/mL).

3.2.ANTISERUM SELECTION

The antiserum from eight rabbits was evaluated for their titer by indirect non-competitive ELISA and specificity by indirect competitive ELISA. Just one antiserum which exhibited both higher titer and the best inhibition was selected.

3.3.SENSITIVITY AND CALIBRATION CURVE OF THE ELISA

Inhibiting curves were obtained by plotting absorbance against the logarithm of ABZ concentrations. The data were transformed to B/B_0 values, where B is the OD value obtained for a standard solution and B_0 is the OD value obtained for zero competitor control in the assay. In range of 1 μg/L–1000 μg/L, B/B_0 values were linear to ABZ concentration, the high relationship (R^2: 0.9946). Based on the calibration curve, IC_{50} was 13.80 μg/L. In order to determine the LOD, blank sample measurement was repeated 10 times to get mean (1.25) and standard deviation (SD, 0.091). The LOD (1.054 ng/mL) was obtained for the concentration of （A_0–2×SD）on calibration curve.

3.4.VALIDATION OF THE METHOD

When pork, porcine liver, mutton and fish were spiked with 200 μg/kg, 100 μg/kg, 10 μg/kg, 1 μg/kg of ABZ respectively, the recoveries (described in Tables 1 to 4) ranged from 71.0% to 95.7% and RSD from 6.2% to 10.8%. The result shows that the method can meet rapid screening requirements. For the commercial A kit, in range of 100 μg/kg to 200 μg/kg, the measured recoveries were 92.3% to 98.7%, while in range of 1 μg/kg to 10 μg/kg, their recoveries were very poor (Tables 1–4).

TABLE 1. DETECTION OF ABZ IN PORK USING TWO KITS (N=6)

ABZ spiked concentration (ng/kg)	ELISA results (ng/kg)		Recovery %		RSD%	
	CAAS	Commercial	CAAS	Commercial	CAAS	Commercial
200	191.3	196.5	95.7	97.6		
100	89.6	93.3	89.6	93.2	10.8	23
10	80.5	17	80.5	170		
1	0.75	2.5	75.0	250		

TABLE 2. DETECTION OF ABZ IN LIVER USING TWO KITS (N=6)

ABZ spiked concentration (ng/kg)	ELISA results (ng/kg)		Recovery %		RSD%	
	CAAS	Commercial	CAAS	Commercial	CAAS	Commercial
200	180.1	197.2	87.9	98.1		
100	83.5	92.5	83.5	92.5	7.6	21
10	7.9	16.2	76.6	162		
1	0.8	1.9	74.0	190		

TABLE 3. DETECTION OF ABZ IN MUTTON USING TWO KITS (N=6)

ABZ spiked concentration (ng/kg)	ELISA results (ng/kg)		Recovery %		RSD%	
	CAAS	Commercial	CAAS	Commercial	CAAS	Commercial
200	179.2	189.5	89.6	94.6		
100	90.5	94.1	90.5	94.1	6.2	24
10	8.4	15.7	84.0	157		
1	0.79	2.4	79.0	240		

TABLE 4. DETECTION OF ABZ IN FISH USING TWO KITS (N=6)

ABZ spiked concentration (ng/kg)	ELISA results (ng/kg)		Recovery %		RSD%	
	CAAS	Commercial	CAAS	Commercial	CAAS	Commercial
200	175.5	194	87.8	98.2		
100	82.4	92.3	82.4	92.3	8.8	26.4
10	7.92	18.3	79.2	183		
1	0.71	2.9	71.0	290		

3.5.CROSS REACTION

Residues of ABZ, ABZ–SO_2, ABZ–SO, OXI, OFZ and FBZ were measured by the developed ELISA kit. The results (Table 5) implied that ABZ and its two metabolites can be detected the other drugs in the BZ family could not be screened by the ELISA kit.

TABLE 5. THE CROSS–REACTIVITY OF RELATED ANALYTES WITH THE DEVELOPED ELISA

Analyte	Cross–reactivity
ABZ	100%
ABZ–SO_2	92%
ABZ–SO	57%
OXI	13%
OFZ	8.5%
FBZ	4%

4. CONCLUSION

A competitive ELISA was established to screen albendazole and its metabolites in animal foodstuff using ABZ anti–serum. The inhibiting curve of B/B_o to logarithmic concentrations in the 1 μg/L–1,000 μg/L range showed that IC50 was 13.80 μg/L. The average recoveries were 85.2%, 79.4%, 85.8% and 81% for liver, pork, mutton and fish tissue spiked samples, while the % RSDs were 10.8%, 7.6%, 6.2%, 8.8%. The result showed that the method can meet rapid screening requirement for residue survey.

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CONFIRMATORY METHOD FOR DETECTING MULTI–BENZIMIDAZOLES, PROBENZIMIDAZOLES AND THEIR METABOLITES IN ANIMAL PRODUCTS

YANG， S., LIU, H., CHEN, G., ZHIHUA, Y.,
Institute of Quality Standards and Testing Technology for Agro–Products, CAAS, China

Abstract

An efficient and sensitive liquid chromatography and tandem mass spectrometry (LC–MS/MS) method was developed for determination of benzimidazole residues in animal tissues and milk. Using $^{13}C_6$–thiabendazole (TBZ) as internal standard (IS), twelve benzimidazoles (BZs), including parent compounds, their pro-drugs and metabolites, were extracted from samples by alkaline ethyl acetate and excess fat removed with n–hexane. The milk samples were extracted and purified with an optimized QuEChERS method. Using acetonitrile–0.005 mol formic solution as mobile phase at a flow rate of 0.3 mL/min, mass spectrometry was performed in multiple reaction monitoring mode. Blank samples were fortified in the concentration range of 1 μg/kg–200 μg/kg. The ranges of recoveries were between 70% and 110% with coefficient of variation less than 10%. The limits of detection (LOD) were below 0.5 μg/kg and the limits of quantification (LOQ) were below 1.5 μg/kg. Compared with external standard method, this method is more stable and precise.

1. INTRODUCTION

As an important group of broad–spectrum and efficient anthelminthics, BZs are widely used in food producing animals for treating animals infected by gastrointestinal nematodes, lungworms and liver–fluke [1]. However a number of approved BZs have been shown to have teratogenic and embryotoxic problems in some species [2, 3]. Thus residues of BZs and their metabolites are listed for control in many national residues monitoring programs.

Benzimidazoles are metabolized in liver to molecules such as ABZ–SO and ABZ–SO_2 (for ABZ), while others are prodrugs that require metabolism to biologically active BZs (e.g. febantel converted to FBZ). These parent and metabolite BZs are of food safety concern and therefore require control based on maximal residue limits (MRLs) in animal products which, for instance range from 10 μg/kg to 1,000 μg/kg in tissues such as liver, kidney, muscle and fat according to EU legislation and related guidelines [4, 5].

A number of detection methods have been established for BZ residues, including HPLC [6–9], gas chromatography [10] LC–MS/MS [11–13] and immunoassay [14–16]. Biological detection methods and thin–layer chromatography (TLC) have also described. However, the sensitivity and specificity of some of these methods (such as TLC) cannot meet the need for trace level detection. Because of the thermal decomposition, GC may also not suitable for quantitation of heat labile analytes. Although HPLC has been used widely for detection of BZ residue, its sensitivity may be inadequate in some cases.

The LC–MS/MS is a more reliable quantification and confirmation method for trace levels of multi residues in a variety of complex matrices. Nevertheless, analytical methods for simultaneous determination of pro–drugs, drugs and their metabolites are seldom reported. This paper therefore describes an LC–MS/MS multi residue method for residues of twelve benzimidazoles as prodrugs, parent drugs and their metabolites in different animal tissue.

2. MATERIALS AND METHODS

2.1. REAGENTS AND STANDARDS

HPLC grade acetonitrile (MeCN), n–hexane, ethyl acetate, methanol (MeOH) and formic acid (JT Baker of USA) as well as demineralized water (H_2O, Milli–Q Gradient, Millipore) were used [17].

The standards used were: ABZ (99.0%), ABZ–SO (98.5%), ABZ–SO_2 (99.0%), ABZ–2–NH_2SO_2 (>99%), thiabendazole (TBZ, 98.5%), 5–hydroxy–TBZ (99.5%), oxibendazole (OXI, 98.5%), oxfendazole (OFZ, 99.0%), fenbendazole (FBZ, 99.0%), fenbendazole sulfone (FBZ, SO_2>99%), febantel (99.0%), thiophenate–ethyl (99.0%) (Dr. Ehrenstorfer, Germany) [17].

Standard stock solutions (100 mg/L in MeOH) and working standard solutions (~10 mg/L, following dilution of the stocks with MeOH) were prepared and refrigerated at 4°C [17].

2.2. APPARATUS

The samples (pork, pork liver，mutton, milk and fish) purchased from supermarkets were homogenized with an IKA T18 ultra–turrax disperser (Germany) mixed with a vortex (Kylin-Bell, Jiangsu, China) followed by centrifugation at 5,000 rpm (Xiangyi instrument Co, China). Organic layers were evaporated suing a rotary evaporator (Eyela, Tokyo Rikakikai Co. Ltd) and the residues dissolved in MeOH on an ultrasonicator (Kun Shan Ultrasonic instrument Co. Ltd, China). After defatting using an organic solvent, the extracts were evaporated using a pressure blowing concentrator [17].

The LC–MS/MS system comprised the 1200 series LC–system (Agilent Technologies, USA) with a G1312B Bin pump system, G1322A degasser and G1367C HIP–ALS autosampler, an API2000 LC–MS/MS (Applied Biosystems, USA) with ESI ion interface. The AB Sciex Analyst software, version 1.4.2 was to control the instrument control and acquire the data acquisition [17].

2.3. INSTRUMENTATION CONDITIONS

2.3.1. HPLC conditions

Chromatographic separation was achieved on a 50 mm × 4.6 mm 1.8 µm particle size, Eclipse × DB–C18 analytical column (Agilent Technologies, USA). The mobile phase was a gradient type prepared from MeCN (15%, v/v) and Milli–Q–H_2O containing 0.005 mol formic acid in which the amount of MeCN was changed linearly as follows: 0 min, 15%; 5 min, 80%; 8.5 min, 80% and 8.6 min, 15% until 14 min. The flow–rate was 0.3 mL/min. Injection volume: 10 µL for both standards and sample solutions and column temperature was 26°C [17].

2.3.2. MS

The API2000 MS equipped with the ion spray interface was used for positive ion detection and multiple reaction monitoring mode (MRM). Nitrogen gas (purity grade 5.0) was the collision, curtain, drying and nebulizing gas, while the optimized ESI (+)–MS/MS operating conditions were: focusing potential: 400 V, entrance potential: 10 V, collision cell exit potential: 4 V, temperature: 450°C，ionspray voltage: 4,500 V, gas 1: 50 psi, gas 2: 60 psi, curtain gas: 30 psi, collision gas: 10 psi, dwell time: 50 ms. The quantitative and qualitative

ions plus related parameters are thoroughly optimized. The cone voltages and energies averaged 43 and 42, respective. The cone voltage of 65 was an outlier [17].

2.4. PREPARATION OF SAMPLES

2.4.1. Extraction and clean–up of muscle and liver samples

To 5 g of homogenized muscle or liver tissues in 50 mL centrifuge tubes, IS, 3 g of sodium sulphate, 3 mL 2M of potassium carbonate and 15 mL of ethyl acetate were added followed by shaking on a vortex mixer for 2 min and centrifugation for 5 min at 5,000 rpm. The supernatant was then decanted into 100 mL distillation flask (extraction with 15 mL of ethyl acetate) and the collected organic phases evaporated to dryness under nitrogen (45°C). Also 3 mL MeCN and 5 mL n–hexane was added to the residue and the content mixed for 2 min in an ultrasonic cleaner, before transfer to a 10 mL centrifuge tube. After centrifugation (5 min, 5000 rpm) the upper layer was discarded and 5 mL n–hexane added for defatted again. The MeCN layer was then evaporated to dryness under nitrogen (45°C), the residue dissolved in 1 mL MeCN, and 100 µL solution pipetted into 900 µL MeCN:H_2O (30:70, v/v). Filtration on PES 0.22 µm before injected into the LC–MS/MS.

2.4.2. Extraction and clean–up of milk samples

Milk samples (5 mL) were measured into 50 mL polypropylene centrifuge tube, the internal standard, 20 mL of MeCN containing 0.5% acetic–acid were added and content shaken on a vortex mixer for 2 min. Anhydrous sodium acetate (1.5 g) and anhydrous sodium sulphate (6.0 g) were then added to each tube and shaken vigorously for 2 min, centrifuged for 5 min at 5,000 rpm, 4 mL supernatant evaporated to dryness under nitrogen (45°C), and the residue dissolved in 2 mL MeOH. Also 50 mg PSA was added followed by vortexing for 2 min and then centrifugation (5 min at 5,000 rpm). An aliquot of 1 mL of the supernatant was evaporated to dryness under nitrogen (45°C) and dissolved in 1 mL MeOH:H_2O (30:70, v/v) followed by filtration on PES 0.22 µm before injection into the LC–MS/MS.

3. RESULTS AND DISCUSSION

3.1. THE OPTIMIZATION OF OPERATING CONDITIONS

Other than the precursors, BZs ABZ, ABZ–SO and ABZ–SO_2 have a common fragmentation pattern including the ions m/z 159 and 191. They may also lose the same groups in ionization process, such as TBZ and TBZ–5–OH [17]. In this case, optimization of the MS/MS conditions was important [17].

Use of high proportion of organic solvent as mobile phase or adjusting pH value of mobile phase [17] prevents potential reaction between BZs and silanols on the analytical column, which could negatively impact separation and detection and chromatographic parameters. The combination of 0.005 mol/L formic acid and MeCN as mobile phase with gradient elution was most suitable [17]. Also a constant volume of MeCN:H_2O (30:70, v/v) was added to standard and samples [17].

3.2. THE OPTIMIZATION OF PROCESSING CONDITIONS

A combination of ethyl acetate and 2 mol/L sodium carbonate solution was found to be an optimal extractant [17] confirming solubility of BZs in polar solvents under alkaline conditions. However, protein in milk matrix is denatured by organic solvent to be gelatinous,

which greatly change volume of extract. The pre–treatment methods for muscle and liver are not suitable for milk matrix. The QuEChERS method widely used in pesticide residual test [18, 19] is however preferred for milk sample pre–treatment. The suitability of the following were determined based on recovery results (and clean–up effect):acetic acid in MeCN at 0.1%, 0.5%, 1%; MeCN, acetone, MeOH and ethyl acetate; C18, PSA, amino–NH_2 and florisil. The combination of MeCN with 0.5% acetic acid, MeOH, PSA yielded better results [17]. Also based on recovery and costs, the combination of 20 mL MeCN, 6 g anhydrous magnesium sulphate and 1.5 g anhydrous sodium acetate, 50 mg PSA was most suitable in sample pretreatment [17].

3.3. METHOD PERFORMANCE

3.3.1. Linearity and limit of detection (LOD) and limit of quantification (LOQ)

Using matrix matched calibration curves based on blank samples (range of 1 µg/kg to 200 µg/kg) suitable linearity ≥ 0.9950 [17]. The LOD and LOQ were ≤ 0.5 µg/kg and 1.5 µg/kg, respectively [17].

3.3.2. Recoveries and precision

Following fortification of pork, mutton, fish, pork and liver samples with the standards 5 µg/kg, 50 µg/kg, 100 µg/kg and 200 µg/kg) mean recoveries ranged from 70% and 110% with coefficients of variation below 10% [17]. Use of ISs improved the method with lower RSD [17]. Additional validation information is reported in Annex I.

4. CONCLUSION

A sensitive and specific method based on LC–MS/MS for simultaneous determination of 12 kinds of BZs in milk and animal tissues was developed and validated. Because of reasons of matrices, the BZ compounds, including their prodrugs and metabolites, were extracted from samples by alkaline ethyl acetate, degreased by n–hexane during the pre–treatment process of animal tissues, and the milk samples were extracted and purified with optimized QuEChERS method. Twelve kinds BZs could be separated well in less than 15 min. Compared with external standard method; this method is more stable and more precise for multi residues analysis of BZs in animal foodstuff meeting with analysis requires of EU regulations.

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ANNEX I.

VALIDATION OF THE ESTABLISHED LC–ESI–MS/MS

Three independent labs were invited to validate the authors' procedures for sensitivity, precision and accuracy with three kinds of matrixes. Those labs are accredited by ISO 17025 and carrying out national residue survey. They are Quality Control and Inspection Centre for Domestic Animal Products of Ministry of Agriculture, Feed Industry Centre of Ministry of Agriculture, and Analytical Centre of Feed Research Institute of CAAS.

I-1. VALIDATION BY QUALITY CONTROL AND INSPECTION CENTRE FOR DOMESTIC ANIMAL PRODUCTS OF MINISTRY OF AGRICULTURE

I-1.1. Linearity, LOD and LOQ

Method linearity was determined using matrix matched calibration curves with concentrations in the range 2 μg/kg to 200 μg/kg. The lowest r^2 was 0.997 while the LODs ranged from 0.06 μg/kg to 0.34 μg/kg.

I-1.2. Accuracy and precision

Pork, mutton and liver samples (n=5) were spiked at 2.5 μg/kg, 50 μg/kg and 100 μg/kg and the recoveries ranged from 75% to 114% with CVs of 2.06% to 8.21% for pork, 83.9%–108% and 0.71%–7.87 % CV in mutton and 71.1%–100 % with CV of 1.34%–19.61% for liver. [17].

I-2. VALIDATION BY FEED INDUSTRY CENTRE OF MINISTRY OF AGRICULTURE

I-2.1. Linearity, LOD and LOQ

Here the matrix matched in range 1 μg/kg to 200 μg/kg produced r^2 values ≥0.9950; the LOD and LOQ were 0.5 μg/kg to 1.5 μg/kg, respectively [17].

I-2.2. Recoveries and precision experiment

With samples spiked at 5 μg/kg, 50 μg/kg, 100 μg/kg and 200 μg/kg, the lowest recovery was 71.8% and the highest 110% while the highest and lowest CVs were 9.57% and 1.69%, respectively [17].

I-3. VALIDATION BY ANALYTICAL CENTRE OF FEED RESEARCH INSTITUTE OF CAAS

I-3.1. Linearity, LOD and LOQ

The r^2 was higher then 0.9970 based in matrix matched calibration curves at 1 μg/kg–200 μg/kg, while the LODs and LOQs were also 0.5 μg/kg and 1.5 μg/kg, respectively [17].

I-3.2. Recoveries and precision experiment

The recoveries and CVs for fish and milk spiked at 5 (or 3 for milk), 50 μg/kg, 100 μg/kg, 200 μg/kg) ranged from 70.2% to 108% (for recoveries) and 2.37%–9.73% for the CVs [17].

DEVELOPMENT AND VALIDATION OF THIN LAYER CHROMATOGRAPHIC METHOD FOR SCREENING OF SULFONAMIDES IN CHICKEN MEAT

PREMARATHNE, J.M.K.J.K., MUNASINGHE, D.M.S., ABEYNAYAKE. P.
Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka

Abstract

A screening method for analysis of five sulfonamides namely Sulfadiazine (SDZ), Sulfathiazole (STZ), Sulfadoxine (SD), Sulfamethazine (SMZ), Sulfaquinoxaline (SQ) in chicken meat has been developed. Samples were homogenized and extracted with ethyl acetate and the extract evaporated to dryness. The residue was dissolved in methanol (MeOH):water (H_2O) mixture and samples defatted using petroleum spirit before analysis by thin layer chromatography (TLC) at 366 nm following treatment with fluorescamine. The limit of deception (LOD) was at 40 ng/g for all residues except sulfadoxine which was at 60 ng/g. Overall, good recovery values were obtained except for SMZ.

1. INTRODUCTION

The broiler meat industry has been successful in Sri Lanka contributing 70% of livestock industry with broiler meat has become the second most common animal protein source in the country [1]. In order to control poultry diseases such as coccidiosis, farmers are inclined to use sulphonamides; the commonly used sulphonamides include SDZ, STZ, SMZ, SD, and SQ [2]. In spite of many awareness programmes on food quality assurance, the occurrence of antimicrobial residues has not been completely eliminated due to non–compliances and unintentional contaminations [3]. Based on this background a need arose to establish a cost effective analytical technique to detect sulphonamide residues in broiler meat. Among the many techniques already available [4], Thin Layer Chromatography (TLC) is one of the simplest and inexpensive techniques that can be used to screen for the presence of chemical contaminants in food, especially in developing countries.

The TLC is a relatively cheap and easy method for screening of antimicrobial residues. There are various TLC methods developed in the world for analysis of sulphonamides. However, these are capable of analysing one or two sulphonamides. Thus, the objective of this study was to establish a TLC method to detect multiple sulphonamides in chicken tissue for residue monitoring in Sri Lanka.

2. MATERIALS AND METHOD

2.1. CHEMICALS AND EQUIPMENT

All chemicals used including ethyl acetate, petroleum spirit, MeOH, acetone and acetic acid were of analytical grade. Flexible silica gel aluminium backed 250 µm layer TLC plates were obtained from Whatman. Fluorescamine was used for the derivatisation of sulphonamides. The TLC plates were viewed using the Camag TLC viewing chamber and scanned using the Camag TLC Scanner.

2.2. STANDARD AND WORKING SOLUTIONS

Sulphonamides standards; namely SDZ, SD, SMZ, STZ and SQ were obtained from Sigma–Aldrich, St. Louis, MO, USA. A stock solution containing 1 mg/mL of the sulphonamides was prepared in methanol except for SDZ and SQ for which 0.1M NaOH was used as the diluent. The stock solutions were diluted in MeOH to obtain the intermediate standard

solution of 100 µg/mL. The stock and intermediate standard solutions were then stored at -20°C and 4°C, respectively. The intermediate solution was diluted in methanol to prepare working standard solutions.

2.3. SOLVENT SYSTEMS

Silica gel plates were activated in MeOH and dried overnight at 60°C. Different mobile phases and wave lengths were studied for the separation and detection of the analytes. The following mobile phase solvent systems were studied for better separation of analytes.

Solvent system 01: Diethyl ether: MeOH solvent system with a second solvent system of dichloromethane:acetic acid solvent system; Solvent system 02: Ethyl acetate:n–butanol: MeOH: aqueous ammonia solvent system; Solvent system 03: Chloroform and n–butanol solvent system.

2.4. SELECTION OF ANALYTICAL WAVELENGTH

Different wave lengths; 275 nm, 366 nm and 410 nm were also examined for optimum detection of the analytes.

2.5. SAMPLES

Poultry muscles samples of were directly obtained from reliable farms/processing plants that did not use antimicrobials during the grower stage and pre–tested using a microbial inhibition test (six plate method) to ensure that they were free of detectable levels of antimicrobials. Such samples were frozen until used as blank matrices. Accurately weighed 3 g of homogenized samples were treated with 150 µL of 2 µg/mL mixed standard solution in order to prepare spike samples at 100 µg/kg. All samples were kept for 20 min at room temperature before extraction.

2.6. EXTRACTION PROCEDURE

Two extraction procedures were evaluated for sulphonamide analysis.

2.6.1. Extraction method 1

For each sample 0.3 mL of 0.1M HCl and 3 mL of distilled water was added and vortexed for 1 min. Then 4.5 mL ethyl acetate was added into each tube, centrifuged at 524 g for 10 min and the supernatant collected into a glass tube. The remaining pellet was again treated with ethyl acetate and repeated the same procedure. The supernatant collected from the both steps was evaporated under a mild nitrogen flow at 55°C. Finally, the dry residue was dissolved in 1 mL of MeOH:H_2O (75:25, v/v) mixture. Thus prepared solution was treated twice with 1 mL petroleum spirit to remove fat. The resulting solution was evaporated under a mild stream of nitrogen at 90°C, reconstituted with 100 µL of MeOH and used for TLC analysis.

2.6.2. Extraction method 2

Dichloromethane (4 mL) was added to 3 g of the homogenate and vortexed for 1 min. The tube was then centrifuged at 524g for 10 min and the supernatant collected into a glass tube. The remaining pellet was treated again with 4 mL of dichloromethane and the same procedure repeated. The supernatant collected from the both steps was mixed and evaporated under a mild nitrogen flow at 55°C. Finally, the dry residue was dissolved in 1 mL of MeOH:H_2O in

75:25 v/v mixture. The prepared solution was defatted twice with 1 mL petroleum spirit, the resulting solution evaporated under a mild stream of nitrogen at 90°C and reconstituted with 100 µL of MeOH followed by TLC analysis.

2.7. EXPERIMENTAL PROCEDURE

Standards and samples (25 µL) were spotted using graduated micro capillaries on activated silica plates (10 × 10 cm). The space between two spots was 10 mm. Then plates were dried using a dryer. The TLC chamber was kept at room temperature (25 ± 2°C) for 30 min until it was saturated with the mobile phase. The loaded TLC plates were developed under one-dimensional linear ascending mode in a 10 cm × 10 cm TLC trough glass chamber (Camag, Muttenz, Switzerland). The plate was then kept in the chamber until the solvent front reached 8 cm from the sample application point. The developed plate was taken out from the chamber and dried using a dryer.

A 0.1 mg/mL fluorescamine solution was prepared in acetone and used to derivatise samples by spraying it fresh. The developed TLC plates were scanned using a Camag TLC scanner operated with win CATS software (V 3.15, Camag).

2.8. VALIDATION OF THE METHOD

2.8.1. Linearity

The calibration curves were prepared by diluting the working standard solution in methanol to furnish final concentrations of 200 ng/g, 150 ng/g, 100 ng/g, 50 ng/g, 30 ng/g and 25 ng/g. Calibration curves were then constructed by plotting peak areas against analyte concentration. The least–squares method was used for the regression analysis of the curves.

2.8.2. Precision

This was evaluated by intra–day and inter–day repeatability. The inter–day repeatability was assessed by analysing standards at three different concentrations 100 ng/g, 150 ng/g and 200 ng/g and repeating the assay six times. The Relative Standard Deviation (RSD %) was then calculated. The inter–day precision was assessed through the same procedure and conditions by repeating in different days.

2.8.3. Specificity

This was determined by analysing 10 blank samples of chicken muscle in order to verify the absence of potential interfering compounds at Rf values of SAs.

2.8.4. LOD and LOQ

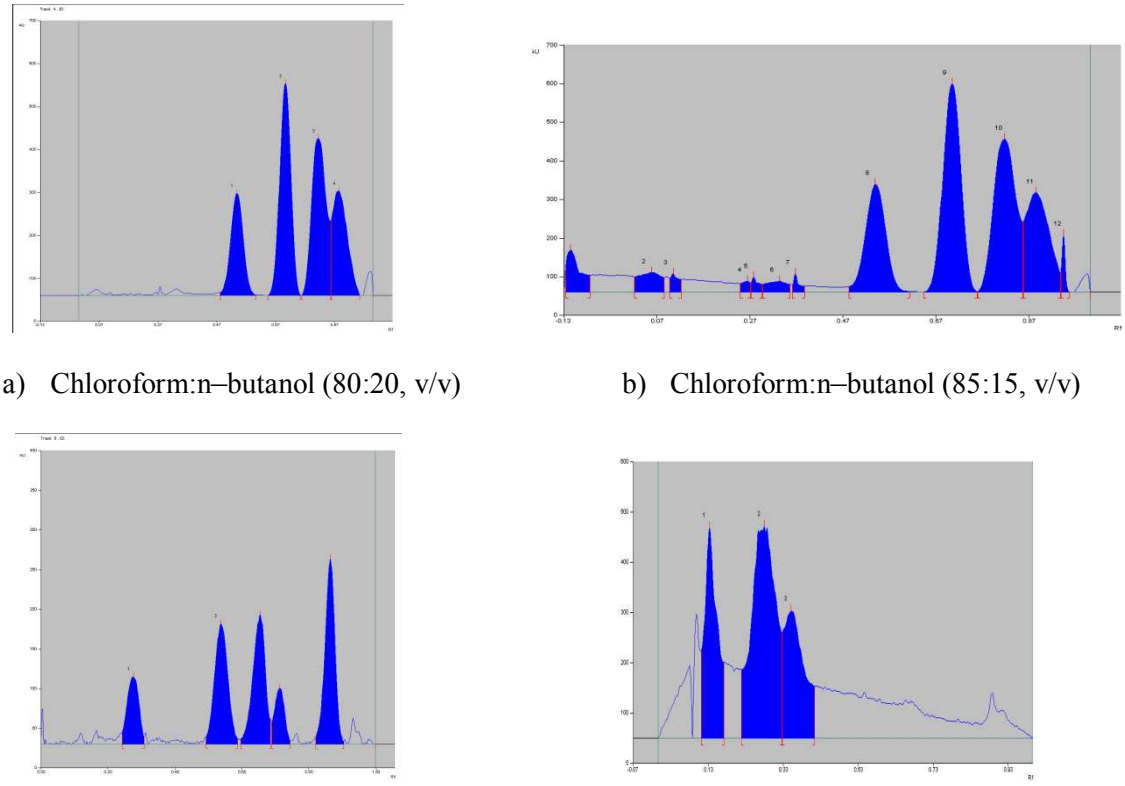
The LOD was calculated using 10 independent sample blanks fortified at lowest acceptable concentration each measured once. Calculation of LOQ was conducted by fortifying sample blank at various analyte concentrations close to the LOD. This was assessed by evaluating the extracts of twenty blank matrixes spiked with sulphonamide mixture at half MRL (50 µg/kg) level.

3. RESULTS AND DISCUSSION

3.1. OPTIMIZATION OF TLC ANALYSIS

The experimental conditions; wave–length, mobile phase and extraction procedure were optimized to obtain more accurate, reliable and reproducible analysis of the sulphonamides in chicken tissue.

Only two separated peaks were visible with the solvent system 01 (diethyl ether:MeOH solvent system followed by dichloromethane: acetic acid). The second solvent system was able to separate the sulphonamide mixture into two separate peaks. The third solvent system containing chloroform and n–butanol at 80:20 (v/v) and 85:15 (v/v) ratios separated the five sulphonamide mixture into four peaks. Meanwhile the solvent combination with 90:10 (v/v was able to separate all five analytes into five separate peaks. The chloroform and n–butanol at 95:5 (v/v) did not resulted in separation between the compounds (Figure 1).



a) Chloroform:n–butanol (80:20, v/v)

b) Chloroform:n–butanol (85:15, v/v)

c) Chloroform:n–butanol (90:10, v/v)

d) Chloroform:n–butanol (95:05, v/v)

FIG. 1. Chromatogram of mixture of sulphonamide standards SDZ, SD, SMZ, STZ and SQ eluted with different combinations of Chloroform:n–butanol and visualized under UV light at 366 nm.

An analysis of the TLC plate applied with sulphonamide standards and spiked samples are shown in figure 2. In this TLC plate, separated five sulphonamides were clearly visible with florescence at 366 nm.

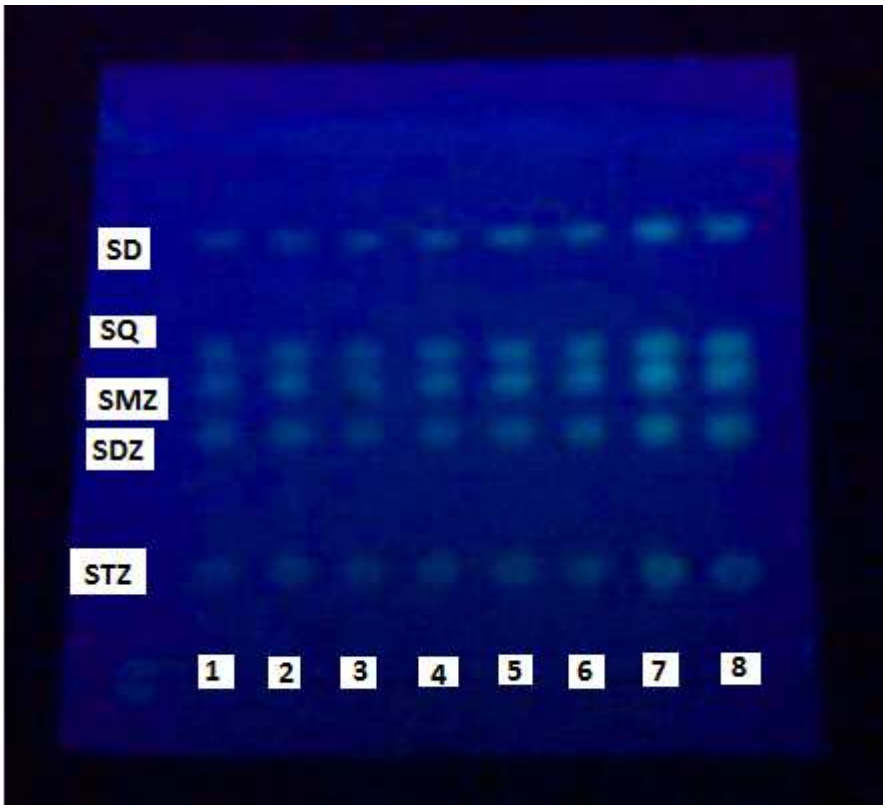


FIG. 2. A TLC plate applied with sulphonamide standards mixture and spiked samples eluted with Chloroform: n–butanol (90:10, v/v) and visualized under UV light at 366 nm. Lane 1:12.5 ng/spot, Lane 2: 25 ng/spot, Lane 3:50 ng/spot and Lane 4, 5, 6, 7, 8 samples spiked at 50 µgkg.

All the chromatograms obtained from scanning at three different wave lengths for triplicates of samples were given in figure 1 (a–d) above. The baseline of the scanned figures at 275 nm and 410 nm was found to be unstable. At 366 nm the sulphonamides showed a higher absorbance and a stable base line.

3.2. OPTIMIZATION OF EXTRACTION PROCEDURE

The mean recovery values obtained with the two different extraction methods are shown in Table 1. The five sulphanomides extracted using method 01 had recoveries within the range of 80%–120% while recoveries for all sulphonamides under the second extraction method ranged from 37%–170% at 100 µg/kg level. The second extraction method with the highest recoveries for all sulphonamides was selected for further studies.

TABLE 1. THE SULFONAMIDE RECOVERY VALUES OF THE EXTRACTION METHODS 01 AND 02

Analyte	Extraction method 01	Extraction method 02
	Recovery %	Recovery %
STZ	81.07	37.8
SDZ	108	96.65
SMZ	120	148
SQ	113	172

3.3. VALIDATION

The calibration curves were established for sulphonamides by plotting peak area against the concentrations. The correlation coefficients for all the analytes were above 0.99. The peak areas of all the sulphonamide analytes were linearly correlated with the concentration range from 12.5 to 100 ng/spot.

The extracts of blank and spiked chicken samples were analysed by TLC (Figure 3). The blank sample in figure 3A illustrates the chicken matrix effect. Figure 3B shows well separated symmetrical and sharp peaks for each sulphonamide in matrix. There were no interfering matrix peaks in the same Rf of the sulphonamides standards.

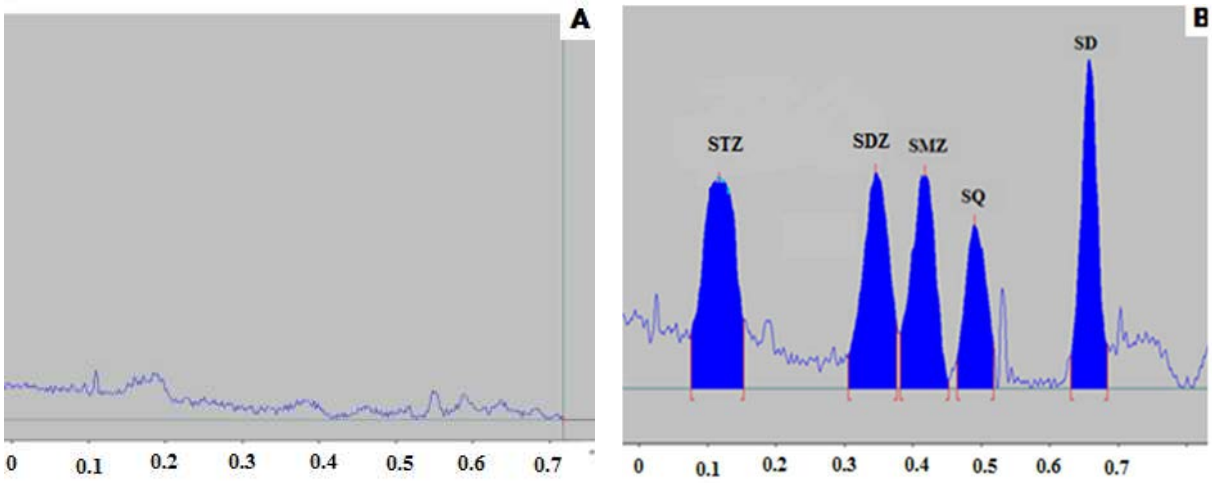


FIG. 3. Chromatograms of blank chicken samples (A), and spiked chicken samples (B) with standard mixture of sulfadiazine (SDZ), sulfadoxin (SD) sulfamethazine (SMZ), sulfathiazole (STZ) and sulfaquinoxaline (SQ) at 100 µg/kg, eluted with Chloroform:n–butanol (90:10, v/v) and visualized under UV light at 366 nm.

The relative standard deviations of intra–day precision were in the range of 3 %–25% for all the sulphonamides (Table 2). Sulfathiazole had the lowest inter day precision of 3.9% at 150 ng/g. Intra–day precision was highest for STZ at 100 ng/g. The highest inter–day precision of 42.6 % was for SMZ and the lowest inter–day precision (6%) was for STZ at 150 ng/g.

The intra–day and inter–day precision of sulphonamides standards at 50 ng/spot, 75 ng/spot and 100 ng/spot eluted with chloroform:n–butanol (90:10, v/v) and visualized under UV light 366 nm.

TABLE 2. THE INTRA–DAY AND INTER–DAY PRECISION OF SULFONAMIDES STANDARDS AT 50 ng/SPOT, 75 ng/SPOT AND 100 ng/SPOT ELUTED WITH CHLOROFORM: n–BUTANOL (90:10, v/v) AND VISUALIZED UNDER UV LIGHT 366 nm

Analyte	Concentration					
	100 ng/g		150 ng/g		200 ng/g	
	Intra day RSD	Inter day RSD	Intra day RSD	Inter day RSD	Intra day RSD	Inter day RSD
STZ	21.90	20.35	3.88	6.64	7.90	19.92
SDZ	17.61	23.54	10.54	12.97	18.40	31.63
SMZ	21.30	27.81	12.35	16.64	14.59	42.60
SD	12.58	26.60	16.82	19.64	16.97	22.12
SQ	13.61	23.78	14.79	30.60	19.44	31.78

Sulfadoxine had the highest LOD and LOQ values of 60 and 100 ng/g, respectively. The LOD of the other analytes was 40 ng/g. Sulfathiazole had the lowest LOQ values of 50 ng/g. The Rf values of the sulphonamides were given in Table 3.

TABLE 3. THE LOD, LOQ AND RETENTION FACTOR OF DIFFERENT SULFONAMIDES IN CHICKEN TISSUE EXTRACTED WITH METHOD 01 AND ELUTED WITH CHLOROFORM: n–BUTANOL (90:10, v/v) VISUALIZED UNDER UV LIGHT 366 nm

Standard	LOD ng/g	LOQ ng/g	Retention Factor
STZ	40	50	0.14±0.018
SDZ	40	60	0.35±0.007
SMZ	40	60	0.43±0.013
SD	60	100	0.48±0.013
SQ	40	80	0.69±0.011

Thin Layer Chromatography is an important method for screening of sulphonamides in animal products. Unruh [5] and Bevill *et al* [6] used methods to analyse only SMZ in milk and plasma, respectively. Reimer and Suarez [7] developed a method for five sulphonamides namely SDZ, SMRZ, SMTZ, SDMX and SP in salmon tissue. A method elsewhere [8] was able to detect SMZ, STZ, SQ, sulfadimethoxine and sulfabromomethazine in beef, pork, turkey and duck meat. Five sulphonamides: sulfanilamide, SDZ, SMTZ, SQ and SD were detected elsewhere in muscle and kidney of pig, poultry and cattle according to Haagsma *et al* [9]. Most of these sulphonamides are not imported or commonly used in Sri Lanka (Drug Registration, DAPH, Sri Lanka). The modified method presented in this study is capable of detecting SDZ, SD, SMZ, STZ and SQ in chicken muscle.

Different types of mobile phases have been developed for separation of sulphonamides. Ethyl acetate:n–butanol:MeOH:aqueous ammonia (35:45:15:2, v/v/v/v) whas been used for separation of five sulphonamides [7]. Fried and Sherma [10] used chloroform: tetra butanol mobile phases to detect SMZ. Haagsma *et al* [9] replaced tetra butanol with n–butanol and were able to separate five sulphonamides. In the current method n–butanol was used in the place of tetra–butanol. In general the first two solvent systems were unable to separate all the five sulphonamides, although the third solvent system we investigated here composed of chloroform: n–butanol was able to demonstrate improved separation. Better separation resulted from increasing the ratio of chloroform. However, when the chloroform percentage increased beyond 95%, there was no separation between the analytes. Haagsma *et al* [9] used chloroform:n–butanol in 4:1 ratio to separate sulfanilamide, SDZ, SMTZ, SQ and SD. However, in this study the sulphonamides (STZ, SMZ, SDZ, SD and SQ) were not properly separated at chloroform: n–butanol ratio of 4:1 (v/v) but at 9:1 (v/v).

The conditions used for the extraction of sulphonamides from different matrices and even different sulphonamides from the same matrices varied from method to method. Reimer and Suarez [7] previously applied Matrix solid–phase dispersion for extraction using C_{18}–derivatized silica gel and eluted sulphonamides with dichloromethane. Unruh [5] employed C_{18} Solid Phase Extraction (SPE) columns for clean–up of samples. Haagsma *et al* [9] also employed Sep–Pak silica disposable columns for the same extraction purpose. This type of extraction is however not affordable in developing countries such as Sri Lanka due to high cost for these SPE columns and unavailability. The SPE columns may provide good recovery values. However, careful market survey has to be done before the employment of such columns in methods used for routine regulatory residue analytical methods to ensure the continuous availability of the columns. Some of the methods employed toxic chemicals like dichloromethane in the extraction procedure. From the studied two extraction methods,

method one was selected due to its high recovery compared with that of the method 02 in which the recovery of STZ was less than 50%. In the current method only ethyl acetate was used for the extraction sulphonamides.

For visualization, different techniques have been employed by different authors. In some methods fluorescamine have been used for the derivatisation [5, 7, 9, 11] while in some methods sulphonamides were detected without derivatisation [8]. In this current study it was observed that intensity of the peak enhanced with the use of fluorescamine, therefore fluorescamine treatment was in cooperated into the method.

The TLC analysis of sulphonamides has been conducted in various commodities of animal origin with different limits of detection. Four sulphonamides namely SDZ, SMTZ, SDMX and SP were analysed at 0.04 ppm and SMRZ at 0.10 ppm in salmon tissue [7]. The method developed by Sigel [11] can only detect SDZ in milk, eggs, liver, kidneys, muscle, skin, and fat at 0.1 ppm. The method reported elsewhere [8] had a LOD more than 0.1 ppm in tissues of cattle, swine, turkey and duck. The LOD of the method by Haagsma *et al* [9] was at 0.05 mg/kg level. In the current method, the LOD was at 40 ng/g for all the sulphonamides except SD which was at 60 ng/g. Nevertheless this method can detect sulphonamides lower than the MRL value and therefore could be applied to detect the five target sulphonamides in chicken meat. Overall, good recovery values were obtained except for SMZ.

4. CONCLUSION

A simples and rapid screening method was successfully developed, optimized for analysis of Sulfonamide (SDZ, STZ, SD, SMZ and SQ) residues in chicken meat. Detection was determined using TLC at 366 nm after treatment with fluroscamine.

ACKNOWLEDGEMENTS

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A HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR QUANTIFICATION OF SULPHONAMIDES IN CHICKEN AND SHRIMP

PREMARATHNE, J.M.K.J.K., * GUNASENA, A.R.C., * FERNANDO, B.R., *MUNASINGHE, D.M.S., ** ABEYNAYAKE, P., *
*Department of Veterinary Public Health and Pharmacology, Sri Lanka
**Department of Basic Veterinary Science, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka

Abstract

A simple liquid–liquid extraction method coupled with high performance liquid chromatography (HPLC)photodiode array detection (DAD) was developed to detect seven sulphonamides; sulphadiazine (SDZ), sulphamethazine (SMZ), Sulphathiazole (STZ), sulphamethizole (SMT), sulphamerazine (SMR), sulphapyridine (SP) and sulphamethoxypyridazine (SMPD), in shrimp and chicken tissues. The tissue was homogenized and extracted with ethyl acetate. The ethyl acetate extract was evaporated to the dryness under a flow of nitrogen and the resulting residue was dissolved in a mixture of methanol (MeOH):acetic acid:water (H_2O (10:4:36, v/v/v). The excess fat was removed using n–hexane and the solution was filtered using a 0.45 µm disposable syringe–filter. The extract was applied to a HPLC system with photodiode array detection and separation was achieved using a mobile phase of 17mM acetic acid:MeOH:acetonitrile (MeCN) (83:10:7, v/v/v) at a flow rate of 1 mL/min and a Luna 5 µm C18 (2) 100A column (250 mm × 4.6 mm). Peaks were detected at 270 nm and peak spectra were collected in the range of 190 nm–400 nm. The total runtime was 30 min. The recovery of the seven sulphonamide residues ranged from 70% to 100% at the maximum residue limit (MRL) of 100 µg/kg, which is within the recommended performance parameters of the EU and Codex Alimentarius. The method is reliable, inexpensive and simple and can be applied to control sulphonamides in animal products and to set standards for antimicrobial residues in food.

1. INTRODUCTION

Sulphonamides are a group of antibiotics used for treatment and prevention of bacterial diseases in humans as well as in animals [1]. In veterinary practice, sulphonamides are extensively used due to its broad spectrum of activity and low cost [2]. Sulphonamides are also used as a feed–additive to promote growth in animals [2].

Improper use of these antibiotics may lead to development of microorganisms which are resistant to antibiotics and occurrence of residues in food of animal origin [2]. Some of the sulphonamides such as SMZ and SMR have carcinogenic effects [3]. Therefore, to ensure consumer safety, the European Union (EU) has set MRL of 100 µg/kg for sulphonamides in food of animal origin [3]. The poultry industry is an important livestock industry in Sri Lanka and contributes to 70 % of the total livestock production. On the other hand, the shrimp industry is one of the main foreign exchange earning aquatic sectors in the country.

Considering the above issues, a method to detect sulphonamides in food of animal origin is of great concern. There are many techniques, including amongst others enzyme linked immunosorbent assay (ELISA), HPLC–DAD, thin layer chromatography (TLC), to detect sulphonamide residues in animal tissues. According to European Council Directive 96/23/EC [4] concerning the performance of analytical methods and the interpretation of results, HPLC–DAD is a suitable confirmatory method to detect sulphonamide residues in food of animal origin. Commission Decision 2002/657, implementing Council Directive 96/23/EC [4] updates the requirements with the limitation that full–scan DAD must be used and specific requirements for absorption in UV spectrometry have to be met to provide the necessary specificity and selectivity for designation as a confirmatory test for group B compounds, which includes the sulphonamides. Most of the HPLC methods published have adopted solid

phase extraction with post– or pre–column derivatisation, which are expensive and laborious [3].

The objective of the study reported here was to develop a simple liquid–liquid extraction method coupled with HPLC–DAD to detect sulphonamides in chicken and shrimp tissues.

2. MATERIALS AND METHODS

2.1. REAGENTS AND CHEMICALS

All chemicals; MeCN, n–hexane, MeOH, and ethyl acetate were of HPLC grade, except anhydrous sodium sulphate and acetic acid which were of analytical grade. De–ionized and double distilled H_2O (filtered through 0.45 µm filter) was used.

2.2. APPARATUS

Liquid chromatography was performed using an Agilent 1100 series HPLC system connected to a DAD (HP/Agilent Technologies, Waldbornn, Germany), using a Luna 5 µm C18, 5 µm, 250 mm × 4 mm analytical column. Data acquisition was controlled by ChemStation software, rev. A. 01.02 (Agilent Technologies, Waldbornn, Germany).

2.3. STANDARD SOLUTIONS

Sulphonamide standards; SDZ, SMZ, STZ, SMT, SMR, SMPD and SP were obtained from Sigma–Aldrich, St. Louis, MO, USA. Stock standard solutions of the seven sulphonamides were prepared by dissolving 10 mg of each sulphonamide in 10 mL MeOH. An aliquot (200 µL) of each stock standard solution was diluted to prepare 10 µg/mL intermediate standard solutions. A working mixed standard solution of 1 µg/mL was prepared by pooling 0.5 mL aliquots of individual intermediate standard solutions and making up to a final volume of 5 mL by adding mobile phase comprising 17mM acetic acid:MeOH:MeCN (83:10:7, v/v/v). All standard solutions were stored in the dark at 4°C.

2.4. SAMPLES

Poultry and shrimp samples were obtained directly from reliable farms and stored under refrigerated conditions until used as blank matrices. The meat samples (chicken and shrimp) were cut into small pieces and homogenized for 5 min at a speed of 13,500 rpm using an ultra–turrax homogenizer. An accurately weighed 1.5 (±0.001) g of homogenate was fortified with 150 µL of working standard solution to prepare 100 µg/kg spiked samples. All samples were kept for 20 min before being subjected to extraction.

2.5. EXTRACTION

For the extraction 4.5 mL of ethyl acetate was added to 1.5 g of homogenate and the mixture was vortexed for 1 min. The tube was then centrifuged at 524g for 20 min. The supernatant was collected into a glass tube and evaporated under a mild nitrogen flow at 55°C. Finally, the dry residue was dissolved in 500 µL of a mixture containing MeOH:acetic acid:H_2O (10:4:36, v/v/v). The prepared solution was extracted twice with 1 mL n–hexane to remove any excess fat. The resulting solution was filtered through a 0.45 µm disposable syringe-filter and transferred to a HPLC vial.

2.6. HPLC–DAD ANALYSIS

A 10 μL aliquot of the sample was injected into the HPLC system by an automatic sample injector. The separation of sulphonamides was performed with a 17mM acetic acid: MeOH:MeCN (83:10:7, v/v/v) mobile phase at a flow rate of 1 mL/min. The HPLC–DAD analysis was performed at 270 nm and peak spectra were collected in the range of 190 nm–400 nm. The calibration curves for each sulphonamide were obtained by injecting 10 μL of 50 ng/mL, 100 ng/mL, 150 ng/mL, 175 ng/mL, 200 ng/mL and 250 ng/mL standard solutions. Measurements were taken in triplicate and the average was calculated. The calibration curve parameters were calculated by linear regression.

3. RESULTS AND DISCUSSION

Good chromatography was obtained for all the seven sulphonamides. The chromatograms obtained for chicken also showed the same trends as those of shrimp.

In order to evaluate the best conditions of the mobile phase and to achieve the optimum separation of the different sulphonamides, different combinations of MeCN, MeOH and acetic acid were tested in both isocratic and gradient modes. In addition, the pH of the mobile phase was also adjusted by changing the acetic acid concentrations. The analytes were well separated in 30 min with sharp and symmetrical peaks when a 17mM acetic acid: MeOH:MeCN (83:10:7, v/v/v) mixture was utilized as the mobile phase in isocratic mode.

The calibration curve was obtained by plotting values of peak area against sulphonamide concentration in the 50 ng/mL–250 ng/mL range. The linear correlation coefficients (r^2) were above 0.99 for all sulphonamides tested. The recoveries of the samples were calculated by comparing spiked samples with recovery control samples. The mean recoveries for chicken were in the range of 72%–86% for all analytes at 100 μg/kg. For shrimp samples, the mean recoveries at 100 μg/kg for all analytes except STZ were above 90%, STZ giving a value of 73%.

The retention times were 8.5 ± 0.07, 10.1 ± 0.1, 11.4 ± 0.1, 13.0 ± 0.1, 19.4 ± 0.08, 20.7 ± 0.3 and 22.8 ± 0.4 min, for SDZ, SMZ, STZ, SMT, SMR, SP and SMPD, respectively for shrimp matrix. The retention times were 8.6 ± 0.02, 10.1 ± 0.01, 11.5 ± 0.05, 13.1 ± 0.2, 19.3 ± 0.07, 20.1 ± 0.1 and 23.7 ± 0.2 min, for SDZ, SMZ, STZ, SMT, SMR, SP and SMPD, respectively for chicken matrix.

Roybal et al. [1] have developed a chromatographic method to detect sulphonamides in shrimp; while Pecorelli et al [5] have developed a method to detect sulphonamide residues in pork, shrimp and chicken meat. Both methods require a solid phase extraction procedure and gradient elution which are expensive and laborious. A solid phase extraction method requires commercially prepared disposable cartridges which are very expensive. Therefore, it is impractical to adopt solid phase extraction methods in developing countries like Sri Lanka. Ultimately the customers will have to bear the cost for analytical testing, and such methods would be unaffordable for them.

A simple method utilizing ethyl acetate as the extractant and a hexane wash to remove fats from the extract was therefore developed, based on a post–column derivatization method developed and validated in the then Agrochemicals Unit at the FAO/IAEA Agriculture and Biotechnology Laboratory [6]. That method used a reaction of the sulphonamides with dimethylaminobenzaldehyde in a post–column reaction coil to enhance the sensitivity for UV

detection and increase the specificity of the method. However, the method requires additional reagents and a second HPLC pump for the post–column derivatization, making it more expensive to run than the method described here, which employs direct UV detection after chromatographic separation.

The recovery of the sulphonamide residues in the method described here ranges from approximately 70% to 100% at 100 µg/kg, the MRL set by the EU for sulphonamides and recommended by Codex Alimentarius for SMZ, and the run time is 30 min. This is comparable with other, more complex, published methods.

The present method is capable of detecting seven sulphonamide residues in both poultry and shrimp without solid phase extraction, gradient elution or post–column derivatization, and can be applied in regulatory control programmes to monitor sulphonamides at the level of interest, the maximum residue limit.

4. CONCLUSION

A reliable, inexpensive, simple, method for the rapid analysis of residues of seven sulphonamides in chicken and shrimp samples, using liquid–liquid extraction and isocratic elution HPLC with photodiode array detection, is described in this study. This method could be applied to control sulphonamides in animal products and to set standards for antimicrobial residues in food. Further, this method is presently being validated according to the European Union's Commission Decision 2002/657/EC in order to test shrimp and poultry meat for sulphonamide residues and to issue residue status certificates for exporters.

ACKNOWLEDGEMENTS

The authors thank IAEA for the technical and financial support of this project.

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HPLC–DAD AND NITROFURAN METABOLITES IN SHRIMPS

FERNANDO, B. R., GUNASENA, A.R.C., MUNASINGHE, D.M.S., ABEYNAYAKE, P.
Department of Basic Veterinary Science, Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Sri Lanka

Abstract

An HPLC–DAD technique for tissue bound nitrofuran metabolites namely 3–amino–2–oxazolidinone (AOZ), 5–morpholinomethyl–3–amino–2–oxazolidinone (AMOZ), semicarbazide (SEM) and 1–aminohydantoin (AHD) is described. Nitrophenyl derivatives (NPAOZ, NPAMOZ, NPSEM and NPAHD) were analysed by HPLC–UV at 275 nm and the anaytes detected below 1 µg/kg. Recoveries were above 100% at spike levels of 1 µg/kg–5 µg/kg.

1. INTRODUCTION

Residues of nitrofurans such as furazolidone, furaltadone, nitrofurantoin and nitrofurazone s well as their metabolites are health concerns and use of the drugs is animals for human consumption disallowed [1, 2]. Various analyses and investigations have been conducted on these compounds including their metabolism [3–5] with reference to a set Minimum Performance Limit (MRPL) of 1.0 µg/kg for nitrofuran metabolites foods such as in poultry and aquaculture products [6, 7].

To support Sri Lanka's shrimp industry and residue testing in general a method to screen nitrofuran metabolites 3–amino–2–oxazolidinone (AOZ), 5–morpholinomethylmethyl–3–amino–2–oxazolidinone (AMOZ), semicarbazide (SEM) and 1–aminhydantoin (AHD)] in shrimps that conforms to EU requirements was studied including optimization of sample preparation and detection method [7].

2. MATERIALS AND METHODS

2.1. REAGENTS AND CHEMICALS

These included analytical/HPLC grade reagents such as HCl, ethyl acetate, Hexane, acetonitrile (MeCN) and deionized H_2O as well as AOZ, AMOZ, SEM and AHD analytical standards [7]

2.2. APPARATUS

An Agilent 1100 HPLC–DAD controlled with ChemStation software with a C_{18} (Eclipse, XDB, 4.6×150 mm, particle size 5 µm) analytical column were used [7].

2.3. SAMPLES EXTRACTION

Minced samples were soaked in MeOH, HCl and 2–nitrobenzaldehyde (2–NBA) before shaking in an incubator [7]. Samples were then adjusted to 7 ± 0.5 with Na_3PO_4 NaOH before extraction with ethyl acetate and defatted with hexane. The evaporated organic portion was then reconstituted in acetonitrile and acetic acid before analysis by HPLC. The analytes were separated after a 27 min run using H_2O, MeCN and acetic acid as mobile phase and detected

at 275 nm [7]. The method was also validated based on EU Commission Decision 2002/657/EC [8].

3. RESULTS AND DISCUSSION

Very good chromatographic separation of the 4 derivatized nitrofuran metabolites detected at 275 nm using the HPLC–DAD was realized. The r^2 values were above 0.99 and the decision limit (CCα), as well as detection capability (CCβ) were between 0.32 µg/kg–0.65 µg/kg and 0.61 µg/kg–1.98 µg/kg, respectively [7]. Recoveries were above 100% [7] and the method was in agreement with previous studies [9] though with improved sensitivity, with good resolution in part due to the mobile selected [10]. The method has applied to screen nitrofuran metabolites in aquaculture and poultry products [7].

4. CONCLUSION

A new cost effective HPLC–DAD method for testing residues of nitrofuran metabolites in shrimp was been developed, validated and applied. The method could detect analytes at 1.0 µg/kg.

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HPLC–UV ANALYSIS OF RESIDUES OF NITROFURAN METABOLITES

SHIMWINO, J. I. *., IIPUMBU L *., SWARTBOOI, W*., AMUNYELA, E*., MUKETE, E*., MVULA, E**., HEDIMBI, M**., KASEYA, Q**., AMAKALI, K**
*Central Veterinary Laboratory (CVL), Windhoek, Namibia
**Chemistry department, University of Namibia, Namibia

Abstract

The fitness–of–purpose of an HPLC–UV method for analysis of nitrofuran metabolite residues in beef was studied with reasonable recoveries. The detection limits (LOD) were between 2.5 µg/kg and 5 µg/kg. Nitrophenyl products were also synthesized for easy detection of the metabolites using the HPLC–UV. This included comparision with commercially procured standards.

1. INTRODUCTION

Nitrofurans are used to treat bacterial and protozoan infections including gastrointestinal infections in poultry and pigs [1, 2] and are used to control mastitis [3–5]. Nitrofurans may be added to animal feeds to prevent bacterial enteritis ensuring adequate growth and feed conversion rates [1, 2]. The compounds (furazolidone, furaltadone, nitrofurantoin and nitrofurazone) are carcinogenic [6, 7] and mutagenic [8, 9] and thus widely generally prohibited in food animals [10, 11]. These nitrofurans are rapidly metabolized [12–15] often into protein products metabolites [16, 17] easily detected when treated with 2–NBA to form nitro–phenyl derivatives. Certain chromatographic techniques and associated detectors [17–19] have limitations and are no longer commonly used [13–15].

Metabolites for furazolidone, furaltadone, nitrofurantoin and nitrofurazone namely 3–amino–2 oxazolidinone (AOZ), 5–methylmorpholino–3–amino–2–oxazolidinone (AMOZ), 1–aminohydantoin (AHD), and semicarbazide (SEM) respectively [20] are used as markers for the nitrofurans, including screening (AOZ and AMOZ) using an immune based technique [21]. Although confirmatory tools such as liquid chromatography–tandem mass spectrometry (LC–MS/MS) are preferred to HPLC–UV, this study was conducted to improved extraction process for better detection by HPLC–UV at acceptable level of 1 µg/kg [22, 23].

2. MATERIALS AND METHODS

2.1. REAGENTS AND CHEMICALS

The following were used methanol (MeOH), ethanol, hexane, acetonitrile (MeCN), ethyl acetate (Rathburn Chemicals Ltd Walkerburn); dimethylsulfoxide (DMSO) and 2–nitrobenzyaldehyde (NBA) (Merck, D–600 Darmstadt); AHD, AMOZ and AOZ, (Witega, Laboratorium, Berlin, Germany); SEM (Fluka Chemica); nitro phenyl 1–aminohydantoin (NPAHD), nitrophenyl 5–methylmonilino–3–amino–2–oxazolidinone (NPAMOZ) and nitro phenyl 3–amino–2 oxalidinone (NPAOZ) (Witega, Laboratorium, Berlin, Germany).

2.2. THE HPLC

A Beckman Coulter HPLC with a UV–Vis and C18 Hypersil columns (250×4.6 mm, 5 µm) were used. Analytes were dected at 300 nm following run time of 13 min at a mobile phase (10mM ammonium acetate buffer and MeCN (30:70, v/v at pH 6.5) at flow rate of 1.3 mL/min.

2.3.NITROFURAN METABOLITE STANDARD SOLUTIONS

Stock solutions (1 mg/mL, expiring after 6 m), working solutions (10 µg/mL and 1 µg/mL, expiring weekly and daily, respectively) were prepared using appropriate solutions including MeCN, MeOH, H_2O and chloroform. Complex working standard solutions were also prepared using ammonium acetate buffer solution (0.01M); MeCN (70:30, v/v) while 2–NBA solution was prepared in DMSO.

2.4.SYNTHESIS, PURITY AND CHARACTERIZATION OF NPAOZ, NPAMOZ, NPAHD AND NPSEM

The metabolites NPAOZ, NPAMOZ, NPAHD and NPSEM were synthesized as described elsewhere [15] with some modifications, and purities determined using an IR spectrometer. The metabolites, namely NPAHD, NPAMOZ, NPAOZ and NPSEM were then characterized and identified by nuclear magnetic resonance.

2.5. SAMPLE EXTRACTION

The extraction process followed a method reported by Leitner et al [24]. Samples collected from abattoirs (20 g) and homogenized (ultra–turrax T25) were sub–sampled (1 g) into 30 mL centrifuge tubes. Standards were added at this point to fortification samples prior to extraction and derivatisation [including addition of water (4 mL) 1M HCl (0.5 mL) and 2–Nitrobenzaldehyde (2–NBA)] over 20 h period. Ethyl acetate was used for extraction by shaking and spinning with a vortex mixer before centrifugation. The organic phase was ultimately analysed using an HPLC following defatting with hexane (later removed), evaporation and reconstitution of the residue in H_2O.

3. RESULTS AND DISCUSSION

Acceptable correlation coefficients (0.98) for NPAHD, NPAMOZ and NPAOZ standard curves were obtained and the LOD for AMOZ (0.75 ng/g) and AHD (0.75 ng/g) and AOZ (1.5 ng/g) in beef established. The recoveries ranged from 50%–90% and RSD of 32%. Recoveries were improved by multiple (three) extractions, adjusting the pH to 7.4 ± 0.02, incleasing derivatization time to 20 h and defatting as well using an acidic mobile phase.

4. CONCLUSION

For the analysis of veterinary drug residues, especially those that are banned, there is a need to apply analytical methods that are fully validated and sensitive enough to reliably detect the residues of interest. A modification of the HPLC method described in this study improved the detection level for AHD and AMOZ inorder to meet the EU MRPL of 1 ng/g.

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A LIQUID CHROMATOGRAPHY–TANDEM MASS SPECTROMETRY ISOTOPE DILUTION METHOD FOR NITROFURAN METABOLITES IN ANIMAL PRODUCTS

FERNANDEZ, A. S.,*, ACHI, M.,**, LONGHI, A***., DI BENEDETTO, N.,***
*Food Technology Institute, INTA–Castelar, Moron, Argentina
**Universidad del Salvador, Campus Pilar, Argentina
***Laboratorio Xenobióticos, Buenos Aires, Argentina

Abstract

A multiresidue liquid chromatography tandem mass spectrometry (LC–MS/MS) method has been developed for the nitrofuran metabolites 3–amino–2–oxazolidinone (AOZ), 5–morpholino–3–amino–2–oxazolidinone (AMOZ), 1–aminohydantoin (AHD) and semicarbazide (SEM) in animal products. This included use of AOZ–d4, AMOZ–d5, AHD–$^{13}C_3$ and SEM–$^{15}N_2$ ^{13}C as internal standards (ISs). Method validation was done following EU guidelines and the decision limits (CCα) and detection capability (CCβ) determined.

1. INTRODUCTION

Furazolidone, furaltadone, nitrofurazone and nitrofurantoin are nitrofuran antibacterial agents (NF) widely used to control gastrointestinal infections in cattle, pigs and poultry. Due to the suspicion of mutagenic and genotoxic drug associated with some (e.g. furazolidone) and the insufficiency of data regarding others (e.g. furaltadone, nitrofurazone and nitrofurantoin), the use of nitrofuran antibiotics is prohibited by many such as European Union (EU) [1, 2].

Betwen 2002 and 2003, prohibited drugs such as nitrofurans and Chloramphenicol (CAP) were detected by the EU in various animal products [3]. This could have been as a result of contaminated feed, environmental contamination (housing) or transfer between animals [4] if not by deliberate administration.

Monitoring residues of NFs using the parent compounds rather than the metabolites is ineffective since the drugs are rapidly metabolized and do not persist in edible tissues [5, 6]. These metabolites may be released in an active form in edible tissues [7] and they remain stable long after storage [8] and thus detectable in tissues for up to 7 weeks after the withdrawal [9, 10].

The metabolized can be derivatised with 2–nitrobenzaldehyde (NBA), extracted from tissues and analysed by LC with ultra violet detection (LC–UV), LC–MS or LC–MS/MS [11–13]. Studies conducted with ^{14}C–labelled furazolidone have shown that the protein–bound residue contains the intact side chain 3–amino–2–oxazolidinone (AOZ) [11, 14]. Other metabolites AMOZ, AHD and SEM for furaltadone, nitrofurantoin and nitrofurazone, respectively have also been reported [15].

Based on the EU established minimum required performance limit (MRPL) of 1 µg/kg for NF detection in food products as [16], the LC–MS/MS is one of the main methodology meeting the lower limits of quantification (LOQ) with the highest degree of certainty based on the EU guidelines [17].

This paper describes the development and validation of an LC–MS/MS multiresidue analytical method for NF residues in foods in the form of nitrophenyl–derivatised protein-bound metabolites.

2. MATERIALS AND METHODS

2.1. REAGENTS AND CHEMICALS

Acetic acid, sodium hydroxide, hydrochloric acid 37%, tri–sodium phosphate dodecahydrate, methanol (MeOH, HPLC grade), ethyl acetate (HPLC grade) and acetonitrile (MeCN, HPLC grade) from Merck Argentina. Water (H_2O, HPLC grade, Sintorgan SA, Argentina). NBA (98% purity), Sigma. AOZ (99% purity), AHD hydrocloride (98% purity), SEM hydrocloride (99% purity), AMOZ (99% purity) and the nitrophenyl derivatives of AOZ, AMOZ, AHD and SEM from Witega, Germany as well as Deuterium, ^{13}C or ^{15}NAOZ–d4, AMOZ–d5, AHD–$^{13}C_3$ and SEM–$^{15}N_2$ ^{13}C.

A solution of 2–NBA (100 mM) was prepared by dissolving 1.51 g in 100 mL of MeOH while trisodium phosphate (0.3M) was prepared dissolving 11.4 g of hydrated sodium phosphate in 100 mL of H_2O. A mixture of 50 mL MeCN, 450 mL of H_2O and 0.5 mL of acetic acid were used to dissolve the sample extract before analysis.

Stock solutions (SNI) of AOZ, AMOZ, AHD and SEM were prepared in MeOH at concentrations of 111.6 µg/mL, 103.8 µg/mL, 110.0 µg/mL and 109.68 µg/mL, respectively, and stored at 4ºC in the dark. Intermediate standard solutions (SNII, 1 µg/mL) were prepared by diluting the stock solutions (1:100) in MeOH and further standard solutions (SNIII) of 52 µg/mL, 52.1 µg/mL 51.31 µg/mL and 51 µg/mL for AOZ, AHD, SEM and AMOZ, respectively, prepared.

Matrix matched standards were prepared by adding 10 µL, 20 µL, 40 µL and 80 µL of the nitrofuran standard solutions (SNIII) to blank sample matrix, corresponding to 0.5 ng/g, 1.0 ng/g, 2.0 ng/g and 4.0 ng/g of each compound in the tissue.

2.2. APPARATUS

2.2.1. LC–MS/MS

A triple quadrupole MS with electrospray ionization (ESI)–positive mode (HPLC Waters 2695 Alliance, MS–MS Micromass Premier XE) was used.

2.2.2. LC Conditions

All separations were performed on a Symmetry C18 column (5 µm, 3×150 mm, Waters) with a Symmetry C18 (20×3.9 mm) guard column. The mobile phase was a mixture of phase A (0.5mM ammonium acetate in H_2O, 0.1%, v/v) and phase B (10% of acid acetic (1% in H_2O) in MeCN). The gradient applied is shown in Table 1.

TABLE 1. MOBILE PHASE GRADIENT

Time (min)	% Mobile phase A	% Mobile phase B
0	90	10
1	90	10
14	55	45
16	10	90
18	10	90
19	90	10
22	stop	

The run time was 22 min at a flow rate of 0.4 mL/min while the column oven was maintained at 40ºC and the injection volume was 50 µL. The elution times were 7.09 min, 11.93 min, 12.15 min and 13.44 min for AMOZ, SEM, AHD and AOZ, respectively.

2.2.3. MS conditions

MS detection was performed in ESI positive ion mode and multiple reaction monitoring (MRM). The following parameters were used: capillary voltage 3.5 kV, cone voltage 40 V, source temperature 120ºC, desolvatation temperature 400ºC, flow rate 500 L/h, cone gas flow 100 L/h. The carrier gas was argon at a pressure of 4.2×10^{-3} bar. Substance identification and quantification was performed by selecting one parent ion and two product ions for each analyte, which is in accordance with the EU guidelines for unambiguous positive identification and quantification of analytes. The precursor/fragment ion combinations, dwell times and collision energies are listed in Table 2.

TABLE 2. MS PARAMETERS

Metabolite	Precursor ions (m/z)	Daughter ions (m/z)	Dwell (s)	Collision Energy (eV)	Window (min)
AOZ	236	104	0.3	20	12.0-15.0
		134	0.3	11	12.0-15.0
AHD	249	134	0.2	10	10.0-14.0
		178	0.2	11	10.0-14.0
SEM	209	166	0.2	9	10.0-14.0
		192	0.2	11	10.0-14.0
AMOZ	335	262	0.3	16	5.0-10.0
		291	0.3	11	5.0-10.0
AOZ–d4	240	134	0.3	11	12.0-15.0
AMOZ–d5	340	296	0.3	12	5.0-10.0
AHD $^{13}C_3$	252	179	0.2	11	10.0-14.0
SEM$^{15}N_2{}^{13}$C	212	195	0.3	12	10.0-14.0

2.3.ANALYTICAL PROCEDURE

Honey samples (1 g) were placed in a 15 mL propylene tube and 10 µL, 20 µL, 40 µL and 80 µL of standard solutions III (50 µg/mL) that corresponded to approximately 0.5 µg/kg, 1.0 µg/kg, 2.0 µg/kg and 4.0 µg/kg of each compound in the tissue, 5 mL of hydrochloric acid (0.2M) and 50 µL of 2–nitrobenzaldehyde (100mM) added. The tubes were vortex mixed for 2 min and incubated at 37ºC overnight before adding 0.5 mL of Na_3PO_4 (0.3M) adjusting the pH to 7.0 ± 0.5with sodium hydroxide (2M) and 500 µL sodium phosphate (0.3M) and mixing on a vortex for 5 min. The samples were extracted two times with 4 mL ethyl acetate using a shaker (20 min) and centrifuged (2,500 rpm, 10 min, 20ºC) before removing ethyl acetate supernatant. The combined ethyl acetate extracts were evaporated under a stream of nitrogen at 45ºC. The residue was dissolved in 300 µL of the mobile phase, vortexed for 30 s and pressed through 0.45 µm filter before HPLC analysis. The procedure was suitable for muscle, milk and eggs samples. For the eggs, the extract was washed twice with 2 mL of hexane to defat.

3. RESULTS AND DISCUSSION

3.1. METHOD DEVELOPMENT

The derivatized honey sample was extracted with ethyl acetate before injecting into LC. The NBA derivatization process of the amino groups of the target analytes (within 15 h including extraction) was performed to improve MS sensitivity. The method was found to be suitable

for accurate determination of the four NF metabolites using a mobile phase including ammonium acetate (10mM) as an ion-pairing agent and acetic acid (1%). The method was validated in terms of range, linearity, precision, accuracy, CCα and the CCβ.

3.2. LINEARITY

Calibration curves were ploted using four points (0.05 µg/kg, 0.10 µg/kg, 0.20 µg/kg and 0.40 µg/kg) over a period of two months (m) as summarized in Table 3.

TABLE 3. LINEARITY FOR THE FOUR NITROFURAN METABOLITES (Y=AX+B)

	AOZ	**SEM**	**AMOZ**	**AHD**
a	0.928	0.205	0.629	0.333
SD	0.013	0.021	0.018	0.009
b	-0.054	-0.000	-0.019	-0.018
SD	0.031	0.005	0.042	0.022
R	0.9855	0.9920	0.9453	0.9461

3.3. ACCURACY AND PRECISION

Samples were spiked at 0.05 µg/kg, 0.10 µg/kg, 0.20 µg/kg and 0.40 µg/kg in three repetitions and the accuracy and recovery determined. The same procedure was repeated after 2 m and involving more than one operator (Table 4). The method accuracy was expressed as the difference between the mean of the measured value and its theoretical value, expressed as a percentage of the theoretical value (equation 1).

$$Accuracy\ (\%) = [(Measured\ concentration - Theoretical\ concentration)/Theoretical\ concentration)]x\ 100 \ldots\ldots (1)$$

TABLE 4. WITHIN LABORATORY REPRODUCIBILITY AND ACCURACY, N=38, N=3 AT EACH FORTIFICATION LEVEL; VALUES TAKEN OVER 2 M

	Nominal concentration (µg/kg)	**S.D.**	**CV %**	**Accuracy %**
AOZ	0.52	0.081	15.62	99.82
	1.04	0.118	11.37	100.22
	2.08	0.213	10.26	99.91
	4.16	0.371	8.93	100.01
SEM	0.51	0.015	2.89	98.49
	1.03	0.023	2.25	100.18
	2.05	0.030	1.47	100.52
	4.10	0.061	1.49	99.88
AMOZ	0.51	0.066	12.90	99.60
	1.02	0.136	13.33	100.50
	2.04	0.245	12.01	99.80
	4.08	0.541	13.33	100.03
AHD	0.52	0.044	8.62	97.91
	1.04	0.061	5.88	95.88
	2.08	0.141	6.78	96.72
	4.17	0.240	5.75	95.02

3.4. CC ALPHA AND CC BETA

The CCα and CCβ values for each of the four metabolites were calculated using equations 2 and 3 and results as presented in Table 5.

$$CC\alpha = intercept + 2.33 \times SD\ intercept \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

$$CC\beta = CC\alpha + 1.64 \times SD\ intercept \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

TABLE 5. CC ALPHA AND CC BETA FOR THE FOUR NITROFURAN METABOLITES

	CCα	CCβ
AOZ	0.02	0.07
SEM	0.01	0.02
AMOZ	0.08	0.15
AHD	0.03	0.07

3.5. STABILITY OF INTERNAL STANDARDS

Over the period of 5 m slopes for fortified standard curves for ADH showed discrepancy. The slope of the AOZ, AMOZ and SEM standard curves exhibited no significant differences with time, but the slope of the AHD curves increased with time. This was probably due to degradation of the AHD–$^{13}C_3$ IS of AHD. Consequently the response factor (Rf), calculated as the ratio between the sum of the two daughter ions and the IS, and the slope of the standard curves increased with time. When the AHD data were recalculated using the IS of AOZ as the reference measure instead of the AHD IS, there was no variability in the slopes of AHD curves over 5 m.

This problem of IS degradation could be overcome by using the AOZ IS (which showed greater stability) as the reference. Samples analyzed in short period (for instance within 2 m) had adequate precision and accuracy with either IS (ADH and AOZ), though the precision at lower levels was higher with AHD–$^{13}C_3$ (Table 6).

TABLE 6. INTRA–LABORATORY REPRODUCIBILITY AND ACCURACY OF THE METHOD FOR AHD USING EITHER AHD–$^{13}C_3$ OR AOZ–D4 AS IS OVER A PERIOD OF 2 M

	Nominal concentration (µg/kg)	S.D.	CV (%)	Accuracy (% recovery)
AHD–$^{13}C_3$	0.521	0.3860	74.09	101.14
	1.042	0.6780	65.07	99.56
	2.084	0.1391	6.67	99.83
	4.168	0.2806	6.73	100.05
AOZ–d4	0.521	0.0449	8.62	97.91
	1.042	0.0613	5.88	95.88
	2.084	0.1412	6.78	96.72
	4.168	0.2396	5.75	95.02

4. CONCLUSION

A multiresidue LC–MS/MS isotope dilution method for the NF metabolites AOZ, AMOZ, AHD and SEM in honey was developed and validated and is suitable for control of nitrofuran drugs in food–animal production. Degradation of the AHD–$^{13}C_3$ used as IS for AHD was observed over a period of 5 m. The problem can be overcome by using the AOZ–d4 IS for both AOZ and AHD. Further research will be required to better understand why AHD–$^{13}C_3$ degrades.

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LIST OF PARTICIPANTS

Abeynayake, P.	University of Peradeniya; Faculty of Veterinary Medicine and Animal Science; Department of Veterinary Para–clinical Studies, Peradeniya Sri Lanka
Antonaz, R.	Ministerio de Ganadería, Agricultura y Pesca (MGAP); Dirección de Laboratorio Veterinarios Miguel C. Rubino (DILAVE); Sección Residuos Biológicos, Montevideo Uruguay
Ben Mansour, A.	Centre National des Sciences et Technologies Nucléaires (CNSTN) Tunisia
Campbell, K.	Institute for Global Food Security, School of Biological Sciences, Queen's University, Belfast United Kingdom
De Brabander, H.	Université de Gent; Faculté de médecine vétérinaire Belgium
Elliot, C.	Department of Agriculture and Rural Development (DARD); Veterinary Sciences Division; Residue Section, and later Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast United Kingdom
Enkhtuya, T.	Ministry of Agriculture and Light Industry, State Central Veterinary Laboratory of Mongolia Mongolia
Fernandez, S.	Instituto Nacional de Tecnología Agropecuaria (INTA); Instituto de Tecnología de los Alimentos, Buenos Aires Argentina
Fodey, T.	Department of Agriculture and Rural Development (DARD); Veterinary Sciences Division; Chemical Surveillance Department, and later Institute for Global Food Security, School of Biological Sciences, Queen's University Belfast United Kingdom
Granja, R.	Laboratorio Microbioticos Brazil
Hartel, A.	Technische Universität München; Institut für Physiologie, Freising Germany
Ildiz, F.	Turkish Atomic Energy Authority (TAEK); Ankara Nuclear Research Centre in Agriculture and Animal Sciences; Animal Nutrition Department, Ankara Turkey
Johnsson, H.	Statens Livsmedelswerk; Chemistry Division 3, Uppsala Sweden
Kanarat, S.	Ministry of Agriculture and Cooperatives (MOAC); Department of Livestock Development; Bureau of Quality Control of Livestock Products; Veterinary Public Health Laboratory, Bangkok Thailand
Kuhn, W. T.	Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH (AGES) Austria
Kwon, J.W.	Ministry of Food and Drug Safety, Busan Regional Food and Drug Administration, Center for Food and Drug Analysis Korea, Republic of
Liu, G.	Chemical Analysis and Physical Testing Center, Shenzhen CDC China

Meyer, H.D. (R.I.P.)	Technische Universität München; Fakultät für Brauwesen, Lebensmitteltechnologie und Milchwissenschaft; Forschungszentrum für Milch und Lebensmittel; Institut für Physiologie, Freising Germany
Montes Nino, A.	Microbioticos Analises Brazil
Murdiati, T.	Research Institute for Veterinary Science (BALITVET); Department of Toxicology, Bogor Indonesia
Murilla, A.G.	Kenya Agricultural Research Institute (KARI), Trypanosomiasis Research Centre Kenya
Nogueira, G.	Universidade Estadual Paulista (UNESP); Faculdade de Medicina Veterinaria Brazil
Orlando, L	Ministerio de Agricultura, Servicio Nacional de Sanidad Agraria (SENASA) Peru
Pace, M.	Ministry of Rural Affairs and the Environment; Food and Veterinary Regulation Division, Marsa Malta
Park, J. H.	Cheju National University; Veterinary Medicine Department, Cheju Korea, Republic of
Pfaffl, W.M.	Technische Universität München Germany
Phylactou, A.	Ministry of Agriculture, Natural Resources and Environment; Department of Veterinary Services; Laboratory for the Control of Food of Animal Origin, Nicosia Cyprus
Shimwino, J.	Ministry of Agriculture, Water and Rural Development; Central Veterinary Laboratory, Windhoek Namibia
St. John, S.	Ministry of Agriculture and Rural Barbados
Stolker, A.A.M.	RIKILT - Wageningen University & Research Centre Netherlands
Swemmer, A.	Agricultural Research Council (ARC); Onderstepoort Veterinary Institute; Division of Toxicology, Onderstepoort, South Africa
Yang, S.	Institute of Quality Standards and Testing, Technology for Agro-Products (IQSTAP), Chinese Academy of Agricultural Sciences (CAAS) China

CONTRIBUTORS TO DRAFTING AND REVIEW

J. J. Sasanya

A. Cannavan

E. G. Nacif

See list of participants above

RESEARCH COORDINATING MEETING

Vienna, Austria: 19–23 October 2009
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Goethe Buchhandlung Teubig GmbH
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Willstätterstrasse 15, 40549 Düsseldorf, GERMANY
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