Food Chemistry 197 (2016) 571–580

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Multiresidue method for the simultaneous determination of veterinary medicinal products, feed additives and illegal dyes in eggs using liquid chromatography–tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 1 January 2014
Received in revised form 14 May 2015
Accepted 18 October 2015
Available online 19 October 2015

Keywords:
Eggs
Multiclass
Multiresidue
Veterinary drugs
Coccidiostats
Dyes
LC–MS/MS

ABSTRACT

A multiclass method was developed for the simultaneous determination of 120 analytes in fresh eggs. The method covers the analytes from the groups of tetracyclines (6), fluoroquinolones (11), sulphonamides (17), nitroimidazoles (9), amphenicols (2), cephalosporins (7), penicillins (8), macrolides (8), benzimidazoles (20), coccidiostats (14), insecticides (3), dyes (12) and others (3). Samples were extracted using 0.1% formic acid in acetonitrile:water (8:2) with the addition of EDTA and cleaned using solid phase extraction with Hybrid SPE cartridges. The chromatographic separation was achieved on C_8 column using mobile phase consisting of (A) methanol:acetonitrile (8:2) – (B) 0.1% formic acid in a gradient mode. Validation results according to the Commission Decision 2002/657/EC are as follows: linearity ($r \geqslant 0.99$), recovery (75–108%), repeatability (CV 1.60–15.9%), reproducibility (CV 2.60–15%), decision limit (CCα 2.25–1156 μg/kg) and detection capability (CCβ 2.04–1316 μg/kg). The presented method was used for analysis of 150 real eggs samples taken from monitoring control program.

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1. Introduction

Eggs are one of the most important foods in many countries. They are not expensive, commonly available and used to produce many other edibles. Moreover, it is a full value, functional food with high concentration of the nutrients. Alimentary components of eggs improve the overall health, cure and prevent animal and human diseases (Kovacs-Nolan, Phillips, & Mine, 2005).

It is well known that nowadays the production of food of animal origin is not possible without using veterinary medicinal products and feed additives. In commercial egg production, where numerous of laying hens are reared on limited area, the illnesses can spread very quickly among the birds. As an example, one of the biggest problems in poultry production is coccidiosis. It is caused by protozoa of the genus *Eimeria* and causes mortalities, poor weight gain and feed conversion ratio and decreases egg production (Tewari & Maharana, 2011). Due to that many feed additives called coccidiostats are used. They are forbidden for use in laying hens in the European Union but despite that their residues are still detected in eggs due to the cross-contamination of feed. The feed can be contaminated on the production line and thus may be unintentionally administrated to non-targeted animals. Also, the veterinary medicinal products like antibiotics or insecticides are used to fight infectious diseases and ectoparasites. Moreover, antibiotics might be used illegally for fattening purposes, because of their anabolic effect, or to prevent the outbreaks of diseases. Medicinal products may possess negative effects on animals' and humans' health. They might be carcinogenic, genotoxic, immunotoxic and potentially develop the antibiotic resistance (Liu, Hei, He, & Li, 2011; Robert et al., 2013; Sørum and L'Abée-Lund, 2002; Tewari & Maharana, 2011). Due to those facts European Union countries make efforts to protect public health and monitor the residual level of harmful substances in products of animal origin to keep them on toxicologically acceptable levels. The maximum residue limits (MRL), maximum levels (ML) and maximum residue performance limits (MRPL) for veterinary medicinal products are listed in regulatory documents and guidelines (CRL's (Anon, 2006), 124/2009 (Commission Regulation No 53 124/2009, 2009) and 37/2010 (Commission Regulation No 37/2010, 2009), Regulation No, 54 84/2012 (Commission Implementing Regulation No 84/2012, 2012).

The other aspect directly related to eggs is their colour. Those with intense yellow-orange hue are the most desired by consumers because their freshness and quality is very often associated with their colour (Dvořák, Suchý, Straková, & Kopřiva, 2012). There are only eight dyes allowed for poultry treating, registered as feed additives (List of the authorised additives in feedingstuffs, 2004) but practice shows that besides the dyes that are authorized for

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http://dx.doi.org/10.1016/j.foodchem.2015.10.076
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use, food producers' use also banned substances, which are often very hazardous. These substances include, among others, Sudan dyes, which were detected in eggs in 2006 (www.china.org.cn/english/health/189567.htm). They are industrial dyes used for colouring plastics and other synthetic materials. Sudan dyes are banned as food additives but are relatively often detected in such products like: chili powder and chili products, curry, curcuma, red pepper and virgin palm oil (Rapid Alert System for Food and Feed, RASFF). Sudan dyes belong to the azo-dyes group, which are stable in the conditions in which food is prepared, but they can be enzymatically transformed to carcinogenic aromatic amines in human body (Pratt, Larsen, Mortensen, & Rietjens, 2013).

Despite there are multiclass multiresidue screening methods for even over hundreds of analytes in variety of matrices (Kaufmann, Butcher, Maden, & Widmer, 2008; Peters, Bolck, Rutgers, Stolker, & Nielen, 2009; Qiao et al., 2011) which cover the antibiotics and coccidiostats, there are still not many methods suitable for analysis of egg samples. Quantitative multi-multi methods which fulfil the criteria of the Decision 2002/657/EC for eggs were already reported (Błądek, Posyniak, Gajda, Gbylik, & Żmudzki, 2012; Garrido Frenich, Aguilera-Luiz Mdel, Martínez Vidal, & Romero-González, 2010) and they cover several dozen of analytes. Moreover, there is no multiclass multiresidue method including dyes banned for use in egg production, although multiresidue methods for banned azo-dyes in eggs exists (He et al., 2007; Li, Yang, Zhang, & Wu, 2009; Liu et al., 2011; Qiao et al., 2011).

The aim of this study was to develop a method suitable for the analysis of a wide range of veterinary drugs, coccidiostats, illegal dyes and antiparasitic agents (insecticides) potentially contaminating the eggs destined for human consumption. Further, the method was applied on real eggs samples collected by the Veterinary Inspection. So far, in Poland there was no study for the presence of illegal dyes in eggs. Moreover, by the authors' best knowledge, there is also no published multiclass-multiresidue confirmatory method covering among others banned Sudan azo-dyes in its spectrum of analytes.

2. Experimental

2.1. Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), acetone, chloroform, formic acid (99.5%) (HPLC grade) were provided by J.T. Baker (Center Valley, PA, USA). Dimethyl sulfoxide (DMSO) was obtained from Sigma–Aldrich (Steinheim, Germany). Disodium versenate dihydrate (Na_2EDTA) was from POCH (Gliwice, Poland) and heptafluorobutyric acid (HFBA) was from Fluka Sigma–Aldrich (Munich, Germany). Water was purified through a Mili-Q plus system from Millipore (Bedford, MA, USA). The SPE cartridges Hybrid-SPE^{TM} (30 mg/1 mL) and PVDF syringe filters (0.45 μm, 13 mm) were received from Restek (Bellefonte, PA, USA).

Analytical standards of ampicillin, penicillin V, penicillin G, oxacillin, cloxacillin, nafcillin, dicloxacillin, ceftiofur, cephalexin, cefquinome, cefalonium, cefapirin, cefoperazone, cefazoline, sulfaphenazole, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfamonomethoxine, sulfadoxine, sulfaquinoxaline, sulfadimethoxine, sulfathiazole, sulfaguanidine, sulfadiazine, sulfapyridine, sulfamethizole, sulfachlorpyridazine, sulfacetamide, sulfisoxazole, dapsone, tylosin, erythromycin, tilmicosin, josamycin, azithromycine, roxithromycin, spiramycin, leucomycin, danofloxacin, difloxacin, enrofloxacin, ciprofloxacin, ciprofloxacin d_8, flumequine, sarafloxacin, marbofloxacin, norfloxacin, oxolinic acid, nalidixic acid, orbifloxacin, chlortetracycline, tetracycline, doxycycline, oxytetracycline, metacycline, demeclocycline, streptomycin, dihydrostreptomycin, gentamycin, paromomycin, spectinomycin, kanamycin, neomycin, lincomycin, tiamulin, tulathromycin, trimethoprim, fenbendazole, thiabendazole, hydroxyl thiabendazole, mebendazole, flubendazole, oxibendazole, thiamphenicol, florfenicol, florfenicol amine, sudan I, sudan I-d_5, sudan II, sudan III, sudan IV, sudan red 7B, sudan red G, sudan orange G, citrus red, butter yellow, toluidine red, para red, canthaxantin, as well as dinitrocarbanilide, maduramicin, monensin, narasin, nigericin, robenidine, salinomycin, ethopabate, lasalocid standard solution 100 ngl^{-1}, dimetridazole, hydroxymetronidazole, metronidazole, menidazole, were purchased from Sigma–Aldrich (Munich, Germany). Decoquinate-d_5, dinitrocarbanilide-d_8 and robenidine-d_8, ipronidazole, hydroxyipronidazole, fenbendazolesulfoxide, albendazole, albendazolesulfone, albendazolesulfoxide, hydroxymebendazole, triclabendazolesulfone, triclabendazolesulfoxide, ketotriclabendazole, triclabendazole d_3 were obtained from Witega (Berlin, Germany), and decoquinate from U.S. Pharmacopeial Convention (Rockville, USA). Clazuril, diclazuril, halofuginone, methyldiclazuril and semduramicin were donated from European Union Reference Laboratory (EURL) in Berlin. Fenbendazolesulfone and triclabendazole were obtained from National Measurement Institute (Australia), Aminomebendazole was purchased from Merck (Darmstadt, Germany), cambendazole was obtained from Janssen-Cillag (Neuss, Germany) whereas carnidazol and tinidazol were from Riedel-de Haën (Seelze, Germany). Phoxim, propoxur and carbaryl were purchased by Dr. Ehrenstorfer (Augsburg, Germany).

2.2. Preparation of standard solutions

Coccidiostats stock standard solutions (1000 μg ml^{-1}) were prepared by weighing of 10.0 mg of reference standard and dissolving in 10.0 ml of solvent. Clazuril, diclazuril, dinitrocarbanilide, dinitrocarbanilide-d8, methyldiclazuril, robenidine and robenidine-d8 were dissolved in DMSO, halofuginone in acetonitrile–water (50:50, v:v), decoquinate and decoquinate-d_5 in acetonitrile with formic acid addition. The rest of stock standard solutions were prepared in acetonitrile. The stock standard solutions (1000 μg ml^{-1}) of benzimidazoles were prepared by weighing of 10.0 mg of substances and dissolving in DMSO. The stock standard solutions (1000 μg ml^{-1}) of macrolides, tetracyclines, quinolones, sulfonamides, amphenicoles, insecticides, tiamulin, lincomycin and trimethoprim were prepared by weighing appropriate amount of substances and dissolved in methanol, nitroimidazoles, citrus red, sudan I, sudan II, sudan orange G and sudan red G in acetonitrile, whereas β-lactams and butter yellow were dissolved in ultra-pure water. Canthaxanthin was dissolved in chloroform and sudan III, sudan IV, sudan red 7B, toluidine red and para-red in acetone. All of the solutions in the concentrations of 1000 μg ml^{-1} were kept in the dark below −18 °C for six months.

Working standard solutions at concentrations of validation level were prepared for each group of analytes by dissolving appropriate amount of stock standard solutions in acetonitrile (nitroimidazoles, benzimidazoles, coccidiostats, dyes), methanol (tetracyclines, sulphonamides, fluoroquinolones, macrolides, amphenicoles, insecticides, lincomycin, tiamulin, trimethoprim) or water (β-lactams, IS mixture). A mixed working standard solution used for the sample fortification was prepared by the dilution of 1 ml of each working standard solutions in water up to 10 ml. A mixed solution of internal standards was prepared separately. Working standard solutions were kept in the dark below −18 °C for six months, while the mixed working standard solutions were kept in the dark at +2 to +8 °C for three months.

2.3. Liquid chromatography-mass spectrometry

The LC–MS/MS system consisted of an Agilent 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump, a degasser, an autosampler, a column heater, a switching valve (Valco Instruments Co., Inc., USA) and a triple quadrupole mass analyser QTRAP® 5500 (AB Sciex, Canada). The experiments were carried out in the positive and negative ion electrospray mode (polarity switching mode). The Analyst 1.5.2 software controlled the LC–MS/MS system and processed the data. The Turbo Ion Spray source was operated at 400 °C with the capillary voltage set at 4500 V and −45,000 V. Nitrogen was used as a nebuliser gas, curtain gas and collision gas. The chromatographic conditions tested were:

A. Halo® C_{18} analytical column (2.1 × 150 mm, 2.7 μm) (Advanced Materials Technology, Inc., USA) with a guard cartridge of the same material (4 × 2 mm), operated at 35 °C. The mobile phases consisted of I. (A) methanol:acetonitrile (8:2) – (B) 0.1% formic acid and II. (A) acetonitrile – 0.025% (B) heptafluorobutyric acid. The gradient I. was: 5% A (0–2 min), 95% (2–12 min, held to 25 min), 5% A (25–33 min) and II. 5% A (0–2 min), 90% A (2–11, held to 14 min), 5% A (14–33 min). The flow rate 250 μl min^{-1} at ambient temperature and the injection volume was 20 μl.

B. Halo® C_8 analytical column (10 mm × 2.1 mm, 2.7 μm) (Advanced Materials Technology, Inc., USA) with a C_8 guard cartridge (4 × 2 mm) operated at 35 °C. The mobile phases consisted of I. methanol:acetonitrile – 0.1% formic acid and II. acetonitrile – 0.025% heptafluorobutyric acid. The gradient I. was: 5% A (0–1 min), 95% (1–10 min, held to 20 min), 5% A (20–25 min) and II. 10% A (0–2 min), 90% A (2–11, held to 20 min), 10% A (20–25 min). The flow rate I. was 300 μl min^{-1} and II. 250 μl min^{-1} at ambient temperature and the injection volume was 20 μl.

The mass spectrometer working parameters (ionisation mode, capillary voltage, source temperature, sheath gas flow, nebuliser pressure, fragmentary voltage and collision energy) were optimised both with direct infusion of each standard solutions (0.1 μg ml^{-1}) from a syringe pump at the rate of 7 μl min^{-1} and with a LC-injection. The fragmentation reactions (transitions) used for monitoring were selected on the basis of their significance in the product spectra. The analytes were quantified using multiple reactions monitoring (MRM) mode. For each analyte at least two transitions were monitored, whereas for internal standards one transition was monitored.

2.4. Sample preparation equipment

A homogenizer Polytron PT-3100 (Kinematica, Luzern, Switzerland) operating at 7000 rpm was used to homogenise the egg yolk and albumen. Samples were weighed in 50 ml Nunc™ conical sterile polypropylene centrifuge tubes (Thermo Fisher Scientific Inc., Waltham, MA, USA) and the Waters (Milford, MA, USA) SPE chamber was used for sample clean up. An ultrasound Sonorex (Bandelin electronic, Berlin, Germany) and rotator Stuart STR 4 (Bibby Scientific Limited, Stone, Staffordshire, UK) were used to support the extraction. A centrifuge operating at 4500 rpm, 4 °C MPW-6K15 (MPW Med. Instruments, Warsaw, Poland) was used to remove the precipitated proteins. A VLM Eva EC1/EC2L (VLM GmbH, Bielefeld, Germany) nitrogen evaporator operating at 45 °C was used for sample evaporation.

2.5. Sample preparation

Homogenised eggs samples (2.0 g) were fortified with 10, 20, 30, 60 μl of mixed working standard solution consisting of all analytes and 20 μl of IS mixture. An amount of 8 ml of the solution of 0.1% formic acid in acetonitrile:water (8:2) was used as an extraction solution and 500 μl of 0.1 M EDTA was added. The samples were rotary shaken for 10 min and after that ultrasonicated for 15 min. After the centrifugation (10 min, 4500 rpm, 4 °C) the samples were passed through Hybrid SPE cartridges, preconditioned with 200 μl of 0.1% formic acid in acetonitrile. Additional 1 ml of 0.1% formic acid in acetonitrile was passed through the SPE cartridges and the samples were collected to a glass tube.

2.5.1. Sample pre-treatment for the method optimisation

The resulted extract was divided into two parts (3 ml each) and evaporated. After the evaporation to dryness in nitrogen evaporator at 45 °C all the samples were reconstituted either with 250 μl 0.1% formic acid in water or 250 μl 0.025% heptafluorobutyric acid and filtered through 0.45 μm PVDF syringe filters. Then 20 μl of the samples were injected into LC–MS/MS system for analysis.

2.5.2. Sample pre-treatment for the method validation

The extract was collected to a glass tube for evaporation (N_2, 45 °C). The dry residues were reconstituted in 500 μl of 0.1% formic acid in water, filtered through 0.45 μm PVDF syringe filters. Then 20 μl of the samples were injected into LC–MS/MS system for analysis.

2.6. Method validation

The procedure was validated according to the European Decision 2002/657/EC [1]. The method characteristics such as: linearity, recovery, repeatability, reproducibility, decision limit and detection capability were specified. Six points matrix matched calibration curves spiked at the levels 0, 0.1, 0.5, 1.0, 1.5 and 3.0 times of the validation level (VL) were obtained by plotting the response of respective analyte/internal standard peak area ratio versus the analyte/internal standard concentration. VL was set as the MRL, ML or MRPL level if established and 10 μg/kg for other compounds (except for amoxicillin – 30 μg/kg).

The method precision (repeatability and within laboratory reproducibility) was evaluated by the repeated analysis of egg samples ($n = 6$) fortified with analytes at concentrations of 0.5, 1.0, and 1.5 times of the validation level during one day and was repeated other two days. The recoveries were evaluated in the same experiment as repeatability by comparing the measured concentrations to the fortified concentrations of the samples and the CCα and CCβ were determined by the matrix calibration curve procedure. To evaluate possible interferences in the method, the specificity was established by analysing twenty different blank eggs samples.

The stability of the analytes was taken from the reference laboratories recommendations (EURLS) and from our laboratories experience practice. The stability of the analytes in the extract was checked as follows: after the sample preparation, the resulted extract was collected in a glass tube and kept in the refrigerator at +4 °C for 24 h in the dark. The next day it was reconstituted in the final test solution and injected into LC–MS/MS system. The stability of the analytes in the final test solution was tested after storage of the final solution at +4 °C for 24 h in the dark followed by injection into LC–MS/MS system.

3. Results and discussion

Most of the techniques used in multiresidue analysis of veterinary drugs, coccidiostats and dyes in eggs are liquid-chromatography methods coupled with different detection systems like: UV (Bistoletti, Moreno, Alvarez, & Lanusse, 2011; Qiao et al., 2011), diode array (DAD) (Furusawa, 2011; Gigosos et al., 2000), fluorescence (FLD) (Gajda, Posyniak, Zmudzki, Gbylik,

& Bladek, 2012; Jiménez, Companyó, & Guiteras, 2011a) and mass spectrometry (MS) (Galarini, Fioroni, Moretti, Pettinacci, & Dusi, 2011; Lu, Shen, Dai, & Zhang, 2011). In recent years, mass spectrometry detection became a method of choice in residue analysis of food. Coupling of high-performance liquid chromatography with triple quadrupole mass spectrometer detector provides high specificity and sensitivity compared to HPLC with conventional detection or LC–MS. Especially combining ultra-high pressure liquid chromatography (UHPLC) with tandem mass spectrometry (MS/MS) provides adequate sensitivity for the determination of banned substances (Robert et al., 2013). Although the time-of-flight mass spectrometer (ToF-MS) was also used as detection system of veterinary drugs in eggs (Peters et al., 2009), high resolution mass spectrometry often cannot replace a sufficient sample preparation.

To develop the presented method, covering 120 of analytes, both the sample preparation step and chromatographic conditions were optimized. The performance limits for the veterinary medicinal products were set as MRL, ML, MRPL values (if they exist) and for others as 10 μg/kg. Such choice was dictated by the fact, that there are not many veterinary medicinal products allowed for use in laying hens and due to that compounds without established limits were treated like banned substances. Whereas canthaxanthin is the only permitted dye with established maximum residue limit (MRL) set as 30 mg/kg (Commission Regulation No 775/2008, 2008), such limit is too high to be included in this residue analysis method by LC–MS/MS and due to that it was included as 10 μg/kg as for the other dyes.

3.1. Optimisation of MS/MS conditions

To select ions and MS/MS parameters for the method, analyses were conducted using both positive (ESI^+) and negative (ESI^-) ionisation. The group of sulphonamides, fluoroquinolones, tetracyclines, macrolides, β-lactams, nitroimidazoles, aminoglycosides, insecticides, lincomycin, tiamulin and trimetoprim were detected in positive ionisation mode. Amphenicoles, coccidiostats, benzimidazoles and dyes were detected in both ESI^+ and ESI^-. The parameters: parent ion (Q1), daughter ions (Q3), dwell time, declustering potential (DP), entrance potential (EP), collision energy (CE), cell exit potential (CXP) and retention times (RT) were selected. Results obtained when using polarity switching mode are presented in Table 1.

3.2. Optimisation of chromatographic separation

Two mobile phases consisting of ACN – 0.025% HFBA and MeOH:ACN 8:2 – 0.1% formic acid were tested. As first C_{18} column was chosen for the tests. There were differences observed between the numbers of analytes retained on a column depending on which of the mobile phase was used for the analysis.

For example thiamphenicol and florfenicol were retained when phase with 0.1% formic acid was used while florfenicole amine was not retained on the column (Fig. 1). This compound, as well as aminoglycosides, was separated when standard solution was injected on LC–MS/MS system when mobile phase with ion pairing agent (0.025% HFBA) was used (Fig. 2). The presented results were obtained for standard solutions fortified at MRL value for gentamycin (500 μg/kg) and at 10 μg/kg for florfenicol amine, paromomycin and spectinomycin (banned substances).

Further, because of the high retention times of dyes at C_{18} column, C_8 analytical column was tested for the same chromatographic separations. For the chosen analytes, ion intensities for samples fortified at appropriate level were compared. There were differences observed between the ions intensities depending on which of the mobile phases was used. The selected examples showed that higher intensities for majority of the analytes were obtained when mobile phase containing formic acid was used for the separation (Fig. 3). On the basis of these results, mobile phase consisting of MeOH:ACN 8:2 – 0.1% formic acid was chosen for further evaluation of the method and the final separation of the analytes was conducted on C_8 column.

Because of changing the column, the run time of the analysis shortened from 33 min to 25 min. When the column Halo C_8 was applied, it additionally resulted with better peak shapes, e.g. for amphenicoles (Fig. 1D). The disadvantage of such choice was the loss of florfenicole amine and aminoglycosides, which were not retained on the column.

3.3. Sample preparation

Sensitive multiresidue and multiclass methods have been developed to monitor large number of analytes (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2012; Robert et al., 2013). The main challenge is general sample preparation step, suitable for dozen, or even hundreds of analytes. It is often quite difficult, because of the differences in chemical structures and properties of the analytes, as well as different performance levels. The other problem in LC–MS/MS analysis of a biological sample is a matrix effect caused mainly by the proteins and phospholipids.

Different extraction and clean-up techniques in multiresidue analysis of eggs are useful. Usually, at first, samples are extracted by liquid–liquid extraction (LLE) using organic solvent, e.g., acetonitrile (Li et al., 2009; Spisso et al., 2010) or methanol (Capriotti et al., 2012) to precipitate the proteins and then the solid-phase extraction (SPE) is applied (Furusawa, 2011; Heller, Nochetto, Rummel, & Thomas, 2006). This step utilizes the use of majority types of cartridges. To remove the lipids by liquid–liquid (He et al., 2007; Mortier, Daeseleire, & Delahaut, 2003) or solid-phase extraction (Olejnik, Szprengier-Juszkiewicz, & Jedziniak, 2010) n-hexane is used. The techniques of matrix solid-phase dispersion (MSPD) and pressurized liquid extraction (PLE) (Heller et al., 2006; Herranz, Moreno-Bondi, & Marazuela, 2007) were applied to reduce the solvent consumption and time of the analysis. Some authors applied QuEChERS method (“Quick, Easy, Cheap, Effective, Rugged and Safe”), originally developed for pesticides, in the analysis of eggs (Nakajima et al., 2012).

All of the sample preparation steps were tested previously in our laboratory for selected analytes of veterinary medicinal products (Piatkowska, Jedziniak, & Zmudzki, 2014). On the basis of that we chose the most promising procedure to develop and fully validate multiclass-multiresidue method for screening and confirmatory purposes.

In this experiment, a mixture consisting of acetonitrile:water in the proportion 8:2 was used as an extraction solvent. The addition of formic acid was dictated by the manufacturer of Hybrid SPE columns. They are zirconium coated silica columns which allow for the removal of phospholipids, which are present in the egg samples in high content, without any specificity to recovered analytes and their metabolites. Their mechanism of action is a selective Lewis acid base interaction between the zirconia ions bonded to the stationary phase with phosphate moiety of phospholipids. As it was previously reported, the use of 0.1 M EDTA allows to obtain higher recoveries of tetracyclines, because EDTA acts as competing agent for tetracyclines which form chelate complex with metal ions present in eggs sample (Capriotti et al., 2012; Jiménez, Rubies, Centrich, Companyó, & Guiteras, 2011b).

The results of this experiment showed that there were some analytes which were not detected while using designed sample preparation method described above at the expected level, and those analytes were: nimorazole which was recovered at the level of 15 μg/kg, and amoxycilin whose signal was not observed at level up to 30 μg/kg. The method was also found not to be suitable for

Table 1
Tandem mass spectrometry parameters used for the detection and confirmation of the veterinary medicinal products, coccidiostats and dyes. (ESI^+ – positive ionization, ESI^- – negative ionization, RT – retention time, DP – declastering potential, CE – collision energy, IS – internal standard).

Group	Analyte	RT (min)	ParentIon (m/z)	DaughterIons (m/z)	DP (eV)	CE (eV)
ESI^+						
Sulphonamides	Sulfaquinoxaline	9.79	301.3	156/92	70	23/42
	Sulfamethoxazole	8.56	254.3	156/92	54	21/38
	Sulfamonomethoxine	8.46	281.3	156/92	80	25/40
	Sulfamerazine	6.44	256.3	156/92	80	19/33
	Sulfamethoxypyridazine	7.91	281.3	156/92	65	23/40
	Sulfadimethoxine	9.64	311.3	156/92	80	28/45
	Sulfadoxine	8.77	311.3	156/92	80	25/46
	Sulfamethazine	7.61	279.3	108/156	60	25/36
	Sulfatiazole	5.13	256.3	156/92	80	19/33
	Sulfaguanidine	1.09	215.2	156/92	60	20/36
	Sulfadiazine	3.37	251.3	156/92	70	18/30
	Sulfapyridine	5.74	250.3	156/92	100	22/38
	Sulfamethizole	7.60	271	156/92	80	19/38
	Sulfachlorpyridazine	8.25	285.2	156/92	100	20/47
	Sulfacetamide	2.04	215.2	156/92	80	13/30
	Sulfisoxazole	8.93	268.3	156/92	60	19/36
	Sulfamoxole	7.45	268	156/92	80	22/39
	Dapsone	7.97	249.3	156/108	67	21/31
Fluoroquinolones	Sarafloxacin	7.92	386.4	299/342	100	25/39
	Ciprofloxacin	7.29	332.3	314/231	261	28/51
	Enrofloxacin	7.52	360.4	316/245	80	27/38
	Norfloxacin	7.21	320.3	302/231	270	30/54
	Difloxacin	7.89	400.4	356/299	80	28/40
	Danofloxacin	7.45	358.4	340/255	280	32/53
	Flumequine	10.66	262.2	244/202	60	22/44
	Marbofloxacin	6.64	363.3	72/320	100	21/28
	Nalidixicacid	10.50	233.2	215/187	60	19/35
	Oxolinicacid	9.55	262.2	244/216	235	27/39
	Orbifloxacin	7.95	396.3	352/295	56	27/34
Tetracyclines	Doxycycline	7.28	445.4	428/154	150	22/39
	Chlortetracycline	8.60	479.8	445/463	80	31/25
	Oxytetracycline	7.28	461.4	426/443	80	27/19
	Tetracycline	7.31	445.4	410/154	80	27/34
	Metacycline	8.93	443.4	426/201	80	43/23
	Demeclocycline	8.05	465.2	448/430	80	24/31
Macrolides	Josamycin	10.80	828.2	174/229	100	43/76
	Tylosin	10.25	917.1	174/83	100	50/130
	Tilmicosin	9.25	870.1	174/88	100	57/124
	Erythromycin	10.18	734.9	158/83	80	38/96
	Roxithromycin	10.81	838.4	158/680	295	42/31
	Azithromycin	8.69	749.88	83/158	80	100/49
	Spiramycin	8.60	843.5	174/540	120	52/44
	Tulathromycin	8.05	806.6	577.4/158	110	36/52
	Leucomycin	10.55	772.6	174.4/109.2	68	44/54
Penicillines	Cloxacilin	11.67	436	160/277	50	20/20
	Dicloxacilin	12.06	470	160/311	50	20/20
	Ampicilin	7.60	350.1	106/160	58	27/19
	Penicilin V	11.36	351.1	160/114	54	17/48
	Oxacilin	11.43	402	160/243	50	18/18
	Nafcilin	11.85	415	199/171	50	20/50
	Penicillin G	8.77	335.39	91/289	65	72/34
	Amoxycilin	1.41	366.2	114/107	45	37/19
Cephalosporins	Ceftiofur	9.92	524	241/125	100	75/25
	Cefalonium	6.74	459	337/152	46	16/28
	Cephalexin	7.36	348.4	158/106	50	10/23
	Cefquinome	6.81	529	134/125	50	25/75
	Cefapirin	4.46	424	152/124	50	35/70
	Cefazoline	8.52	455	323/156	50	15/23
	Cefoperazone	9.01	646.47	143/530	80	48/15
Nitroimidazoles	Carnidazol	9.35	245.27	118/75	60	21/44
	Ipronidazole	9.00	170.18	124/109	190	32/24
	Hydroxy-ipronidazole	8.06	186.18	168/121	60	19/38
	Tinidazole	6.43	248.27	121/82	60	23/48
	Dimetridazole	3.06	142.13	96/81	70	22/32
	Metronidazole	2.33	172.15	128/82	60	19/34
	Hydroxymetronidazole	1.65	188.15	126/123	60	23/19
	Ronidazole	2.97	201.15	140/55	40	15/30

(*continued on next page*)

Table 1 (*continued*)

Group	Analyte	RT (min)	ParentIon (m/z)	DaughterIons (m/z)	DP (eV)	CE (eV)
	Menidazole	2.15	128.1	82/42	70	24/46
	Ornidazole	8.12	220.625	128/82	60	22/41
	Ternidazole	10.72	186.181	177/149	50	13/28
	Nimorazole	9.40	227.23	114/100	70	20/30
	Hydroxymethylonitroimidazole	2.14	158	140/55	70	16/24
Lincosamide	Lincomycin	6.18	407.5	126/359	283	33/25
Pleuromutilin	Tiamulin	10.03	494.9	192/119	100	17/35
Chemotherapeutic	Trimethoprim	6.58	291.32	230/123	295	32/38
Amphenicole*	Florfenicole amine	1.83	249	231/131	50	16/31
Aminoglycosides**	Neomycine	10.32	615.6	161/163	160	39/43
	Paromomycin	10.32	616.6	163/161	130	47/43
	Spectinomycin	9.26	351	333/207	230	26/31
	Sisomycin	10.25	448	322.3/159.9	100	18/29
	Tobramycin	10.25	468.3	324/163	80	22/30
	Streptomycin	10.31	582.57	263/246	280	43/50
	Dihydrostreptomycin	9.79	584.59	263/246	210	50/41
	Kanamycin	10.06	485	163/205	120	33/33
	Ribostamycin	10.32	455.3	163/295	100	31/24
Benzimidazoles	Flubendazole	10.98	314.28	282/123	280	31/48
	Flubendazole-amine	8.71	256.25	123/95	295	46/66
	Cambendazole	9.08	303.35	217/261	250	39/25
	Mebendazole	10.65	296.29	264/105	270	31/46
	Mebendazole-amine	8.53	238.26	105/133	130	46/81
	Hydroxy-mebendazole	8.77	298.13	266/79	280	34/49
	Fenbendazole	11.59	300.3	268/159	270	31/48
	Fenbendazole-sulfone	10.26	332.35	300/159	280	35/54
	Fenbendazole-sulfoxide	9.79	316.35	159/191	260	45/30
	Albendazole	10.65	266.3	234/191	295	29/45
	Albendazole-sulfoxide	8.77	282.33	240/208	135	20/35
	Albendazolesulfone	9.40	298.33	159/266	60	63/32
	Albendazole-amine	4.22	240.29	133/198	270	39/27
	Oxybendazole	9.16	250.26	176/218	240	39/26
	Thiabendazole	5.81	202.249	175/131	290	37/45
	Hydroxy-thiabendazole	9.08	218.247	191/147	295	35/46
	Triclabendazole	13.08	359.4	274/344	120	37/37
Insecticides	Phoxim	13.00	299.3	77/129	155	44/18
	Propoxur	10.65	210.25	168/111	80	20/11
	Carbaryl	10.89	202	145/127	80	13/13
Coccidiostats	Halofuquinon	9.32	416	120/100	50	29/39
	Decoquinate	13.94	418.5	372/204	290	34/57
	Narasin	15.82	787.3	431/279	50	70/70
	Robenidine	10.81	334	155/138	34	35/47
	Semduramicin	14.33	895.5	833.5/705.5	160	53/85
	Monensin	14.80	693	675/461	60	50/70
	Maduramicin	15.27	934.8	647.4/629.4	170	29/36
	Salinomycin	15.35	773.5	431/531	60	65/65
	Ethopabate	10.10	238.25	206/136	153	16/39
Dyes	Sudan I	13.49	249	232/93	120	25/25
	Sudan II	14.14	277	121/106	100	20/65
	Sudan III	14.49	353	77/156	140	60/30
	Sudan IV	15.35	381	91/224	90	40/30
	Sudan red G	13.47	279	123/108	100	25/50
	Sudan orange G	12.30	215	93/66	140	25/70
	Sudan red 7B	15.11	380	183/115	120	20/70
	Citrus red	13.47	309	153/138	80	20/50
	Para red	13.15	294	156/128	150	20/40
	Toluidine red	13.39	308	156/128	120	40/40
	Butter yellow	13.00	225.5	77/105	60	60/25
	Canthaxanthin	16.90	565.4	133/203	160	40/30
ESI^-						
Coccidiostats	Clazuril	12.20	371	300/301	−120	−24/−24
	Diclazuril	12.59	405/407	334/336	−90	−27/−28
	Diclazuril-methyl	12.68	419	321/333	−140	−40/−40
	Dinitrocarbanilide	12.13	301.24	137/107	−160	−24/−53
	Lasalocid	15.18	589.5	235/173	−140	−46/−67
Amphenicoles	Thiamphenicol	7.36	354	185/290	−120	−32/−19
	Florfenicol	8.80	356	336/185	−80	−14/−27
Benzimidazoles	Triclabendazolesulfone	12.83	389	310/149	−160	−40/−49
	Triclabendazolesulfoxide	12.76	374.66	360/181	−80	−30/−61
	Ketotriclabendazole	12.67	328.56	182/184	−160	−36/−39

Table 1 (*continued*)

Group	Analyte	RT (min)	ParentIon (m/z)	DaughterIons (m/z)	DP (eV)	CE (eV)
Dyes	Carminicacid	8.37	491.2	447.3/327.1	−80	−30/−37
IS	Sulfafenazole	9.48	315.4	158	80	19
	Robenidine d8	10.74	342	182	255	29
	Decoquinate d5	13.94	423	377	290	35
	Nigericin	15.95	747.4	703	10	73
	Sudan I-d5	13.44	254	98	120	40
	Dinitrocarbanilide d8	12.06	309.24	141	−160	−25
	Triclabendazole d3	13.11	361.65	197	−180	−45

* Amphenicole was separated only when the mobile phase contained 0.025% HFBA.
** Aminoglycosides were separated only on Halo C_{18} column with mobile phase containing 0.025% HFBA.

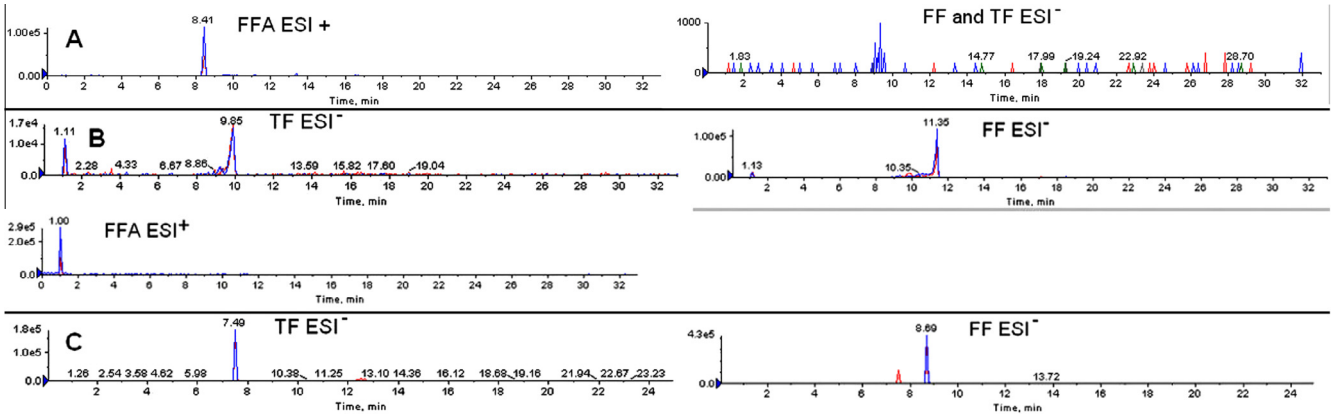

Fig. 1. Chromatographic separation for amphenicols while mobile phases containing heptafluorobutyric acid (A) and formic acid (B) on Halo C_{18} column and heptafluorobutyric acid (C). (FFA – florphenicol amine, FF – florphenicol, TF – thiamfenicol, ESI^+ – positive ionization, ESI^- – negative ionization).

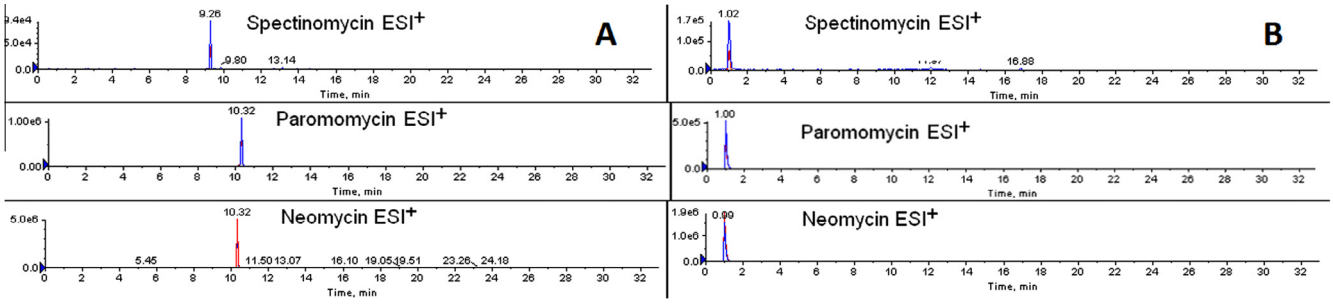

Fig. 2. Chromatographic separation for selected aminoglycosides while mobile phases containing heptafluorobutyric acid (A) and formic acid (B) on Halo C_{18} column.

the extraction of aminoglycoside antibiotics and carminic acid, which were not recovered at all.

3.4. Method validation

The selectivity of the method was checked by analysing blank eggs samples and no peaks were detected in these samples at the retention times corresponding to each analyte. All of the matrix matched calibration curves were linear ($r \geqslant 0.99$) for the determined analytes. The recoveries were in the range of (75–108%). The repeatability was in the range of 1.62–15.9 (CV, %) for the analytes detected with positive ionization and 1.87–6.35% for those recovered in ionization negative, while the within laboratory reproducibility was in the range of 4.97–15% in positive ionization and 2.61–8.51% for negative ionization (supplementary materials). Multiclass multiresidue methods always require a compromise between the number of analytes to be determined and the method precision (Fig. 4).

The CCα and CCβ were in the range of 2.25–1156 (μg/kg) and 2.04–1316 (μg/kg) respectively and fulfilled the requirements of the European Decision 2002/657/EC (2002).

The test of the analytes stability in the extract and final test solution indicated that the analytes kept for 24 h in the dark in +4 °C were stable.

3.5. Analysis of real egg samples

The presented method was used for analysis of 150 real eggs samples taken from monitoring control program. In one case, sample targeted for coccidiostats was found to be non-compliant for enrofloxacin and ciprofloxacin at the concentration level of 94.4 and 9.92 μg/kg, respectively (Fig. 5A). In another sample in which

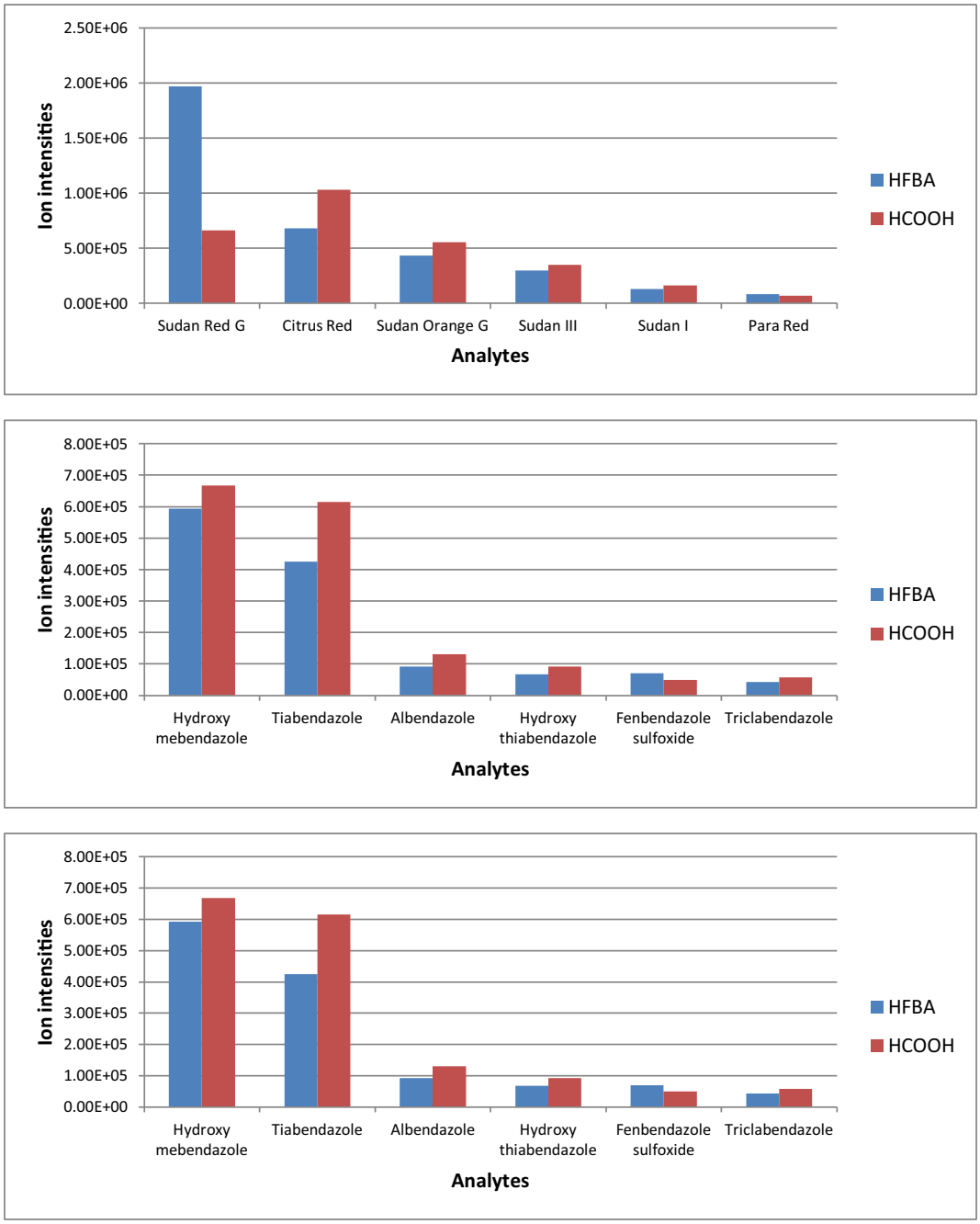

Fig. 3. Comparison of ion intensities for samples fortified at the same level while mobile phases containing heptafluorobutyric acid (HFBA) and formic acid (HCOOH).

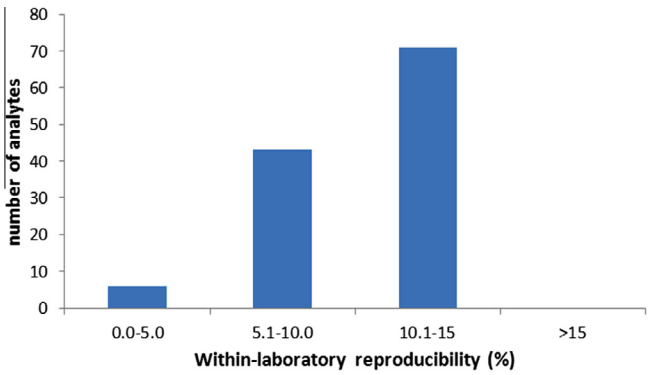

Fig. 4. Ranges of analytes' reproducibility.

lasalocid below the MRL value was present (69.7 μg/kg) doxycycline was found non-compliant (67.8 μg/kg) (Fig. 5B). There were also ten non-compliant samples where the presence of enrofloxacin (21.6–67.8 μg/kg) and doxycycline (5.45–84 μg/kg) were found in eggs samples as a result of investigation procedures taken by the veterinary inspection after detection of antibacterials in water from water supply systems (Gbylik-Sikorska, Posyniak, Sniegocki, & Zmudzki, 2015). All the detected antibiotics are forbidden for use in egg laying hens. The presence of Sudan azo-dyes was not detected.

4. Conclusions

The presented method was found to be suitable for the analysis of residues of veterinary medicinal products, feed additives and

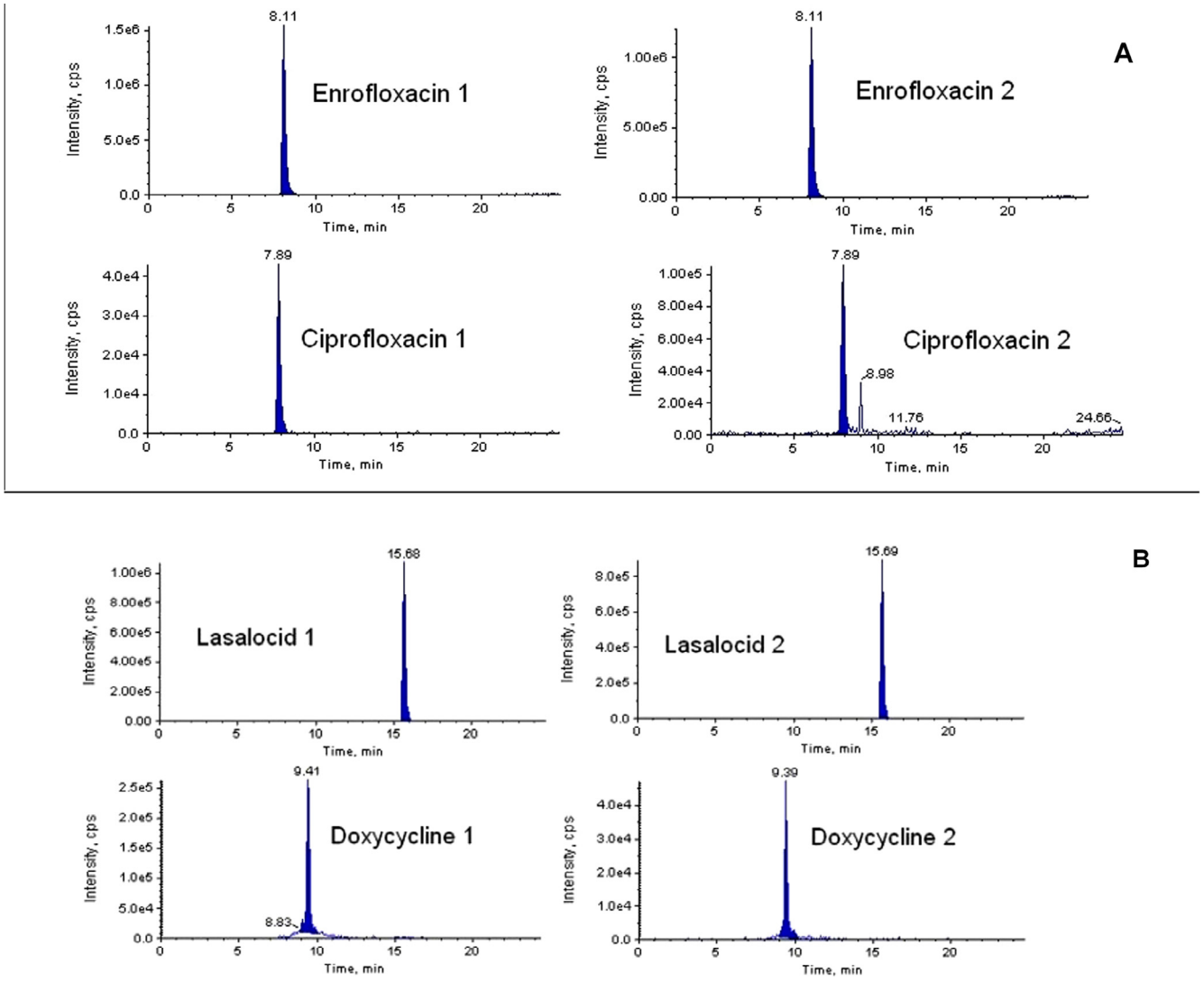

Fig. 5. (A) Sample non-compliant for enrofloxacin at 94.4 μg/kg and ciprofloxacin at 9.92 μg/kg, respectively. (B) Example of sample targeted for coccidiostats with the presence of lasalocid below the MRL value (69.7 μg/kg) and non compliant for doxycycline at 67.8 μg/kg.

illegal dyes in eggs. The results of validation process are satisfactory and the method could be successfully applied in the laboratory both for screening and confirmatory purposes. Multiclass multiresidue methods are more adequate in the official survey of the residues of veterinary medicinal products and feed additives than analysing of targeted samples. In Poland, there is no problem of the contamination of commercial eggs with Sudan azo-dyes banned for use in food production.

Acknowledgments

Many thanks to Alicja Kłopot for invaluable assistance in the preparation of samples for validation. Part of the method including the illegal dyes was supported by National Science Center, Grant Nr 2012/07/D/NZ7/03242 – *"Residues of illegal synthetic dyes in eggs and selected eggs products*". This work was partially financed by the project "*Scholarships for PhD students working in the research teams*" Human Capital Operational Programme, Priority VIII Regional human resources, Measure 8.2 Transfer of knowledge, 8.2.2 Sub-Regional Innovation Strategies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.10.076.

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