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Determination of veterinary drug residues, mycotoxins, and pesticide residues in bovine milk by liquid chromatography electrospray ionisation –tandem mass spectrometry

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Abstract

Introduction: Multi-class and multi-residue analyses are very complex procedures because of the physico-chemical properties of veterinary drug residues and other contaminants. The purpose of the study was to develop an analytical method for the sensitive determination of 69 analytes in bovine milk by liquid chromatography electrospray ionisation–tandem mass spectrometry. **Material and Methods:** Antimicrobial, anabolic hormone, lactone, β-agonist, mycotoxin and pesticide residues were analysed in 120 raw milk samples from different dairy farms in North Macedonia. Stable isotopically labelled internal standards were used to facilitate effective quantification of the analytes. **Results:** The linear regression coefficients were higher than 0.99, the limits of detection ranged from 0.0036 to 47.94 µg/L, and the limits of quantification ranged from 0.053 to 59.43 µg/L. The decision limit values ranged from 0.062 to 211.32 µg/L and the detection capability from 0.080 to 233.71 µg/L. Average recoveries of the analytes spiked in raw milk were in the range of 70.83% to 109%, intra-day coefficient of variation (CV) values from 2.41% to 22.29%, and inter-day CV values from 3.48% to 23.91%. The method was successfully applied in the testing of bovine milk samples. In five samples residues were detected. They were sulfadimethoxine (in two samples), enrofloxacin, tetracycline and oxytetracycline and were at concentrations below the EU maximum residue limit. **Conclusion:** The method is useful for routine testing for this group of chemical hazards in bovine milk.

Keywords: veterinary drug residues, mycotoxins, pesticide residues, liquid chromatography electrospray ionisation–tandem mass spectrometry, validation.

Introduction

In today's global marketplace, food quality and safety have gained increasing attention from consumers, governments and food producers. There are numerous sources of chemical contaminants and residues in food products like pesticides, mycotoxins and veterinary drugs. Most of the analytical methods that have been developed worldwide are based on several targeted analyses in order to cover this broad range of contamination sources and detect as many residues and contaminants as possible. However, such individual hazard-class analysis is very costly and time-consuming and requires the use of larger amounts of consumables. Until now, multi-class liquid chromatography–tandem mass spectrometry (LC-MS/MS) methods have been implemented for determination of individual analyte classes such as antibiotics, non-steroidal anti-inflammatory drugs, anthelmintic drugs, mycotoxins, pesticides in milk or raw milk (4, 12, 13, 19).

While separation and detection techniques are improving, sample extraction and cleanup procedures are still important factors in obtaining reliable results

and maintaining instrument performance. Because milk has high protein and fat contents, which often interfere with analytical procedures, sample extraction and purification require special attention. Multi-class and multi-residue analyses are very complex procedures because of the physico-chemical properties of veterinary drug residues and other contaminants. Therefore, the development of a common extraction procedure and chromatographic conditions is exceedingly difficult. Several methods for the detection of undesirable veterinary drug residues and contaminants such as tetracyclines, quinolones, sulfonamides, hormones, nonsteroidal anti-inflammatory anthelmintic drugs, mycotoxin and pesticides by LC-MS/MS or liquid chromatography time of flight–mass spectrometry in raw milk have been reported (14). The different chemical groups, the amphoteric properties of many compounds, and the wide polarity range pose difficulties for extraction, cleanup, and analytical separation (1, 10, 20). Sample preparation steps include homogenisation, extraction (liquid–liquid extraction or instrumental techniques), clean-up (usually by solid phase extraction SPE), and concentration of extracts. One of the most accepted approaches for milk samples is to dilute a sample with a solvent such as acetonitrile, then centrifuge and evaporate the supernatant's organic extract (9, 22). The number of sample preparation steps should be kept as low as possible to improve the efficiency of the method. The quick, easy, cheap, effective, rugged and safe (QuEChERS) methodology, which was originally developed for pesticide analysis, has been proposed for the analysis of veterinary drugs using different matrices (15, 16). However, low and unsatisfactory recovery of polar veterinary drugs such as penicillin, tetracyclines and quinolones has been reported (15, 16, 20). A low-temperature cleanup method has been developed for these challenging drug residue and contaminant detections in which most lipid components can be successfully separated from extracts. While a wide range of analytes were included, sulfonamides, and several pesticides were not (11, 23, 27).

The aim of this study was to develop an analytical LC-MS/MS method for the sensitive determination of 69 analytes spread across multiple classes including antimicrobials, anabolic hormones, lactones, β-agonists, mycotoxins, and organochlorine pesticides, and to tailor the method to their detection in bovine milk. Different sample treatment methodologies based on SPE and different complexities of extraction (single, double, and triple extraction) were evaluated for the obtaining of the selected chemicals from bovine milk. The methods' performances in terms of linearity, accuracy, intra-day and inter-day precision, limit of detection (LOD), limit of quantification (LOQ), decision limit (CCα) and detection capability (CCβ) were assessed.

Material and Methods

Chemicals and reagents. Methanol, acetonitrile, and LC-MS/MS grade water, ethyl acetate, dichloromethane, ammonium hydroxide, acetic acid, and HPLC grade ammonium acetate were purchased from Carlo Erba Reagent S.A.S (Val de Reuil, France); LC-MS/MS grade formic acid and trichloroacetic acid (≥99.5% purity) were from Merck (Darmstadt, Germany), and Oasis hydrophilic-lipophilic balanced cartridges (500mg/6mL) were supplied by Waters (Milford, MA, USA).

Standards and internal standards. The majority of reference standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), namely amoxicillin (99.6%), ampicillin (99.8%), benzylpenicillin (99.3%), cloxacillin (98.7%), oxacillin (98.4%), lincomycin (100.3%), tylosin (87.9%), trimethoprim (99.5%), tetracycline (96.8%), cefapirin (98.5%), clenbuterol HCl (99.1), isoxsuprine HCl (100%), salbutamol (99.4%), zilpaterol HCl (96.0), ractopamine HCl (95.5%), terbutaline hemisulphate salt (100.0%), taleranol (99.5%), 19-nortestosterone (99.8%), clostebol (99.1%), boldenone (99.1%), methyltestosterone (99.5%), testosterone (100.0%), carbofuran (99.9%), carbaryl (99.9%), parathion (99.7%), malathion (99.2%), diazinon (98.3%), dimethoate (99.8%), atrazine (99.5%), cypermethrin (98.4%), permethrin (98.1%), deltamethrin (99.9%), coumaphos (99.7%), dichlorvos (99.8%), chlorpyrifos (99.8%), boscalid (99.5%), phenthoate (98.8%), fenthion (98.5%), fenvalerate (99.4%), monocrotophos (99.8%), malaoxon (99.0%), methamidophos (98.1%), methacrifos (96.1%), amitraz (99.8%), omethoate (98.4%), vamidothion (≥98.0%), phosmet (99.8%), heptenophos (98.7%), bifenthrin (99.0%) and methomyl (99.0%). Brombuterol (98.0%), mabuterol HCl (98.0%), cimbuterol (98.0%) and clenpenterol HCl (98.0%) were ordered from Witega (Berlin, Germany). Zeranol (99.9%), stanozolol (99.8%), ceftiofur (98.01%), cefalexin (96.6%), oxytetracycline (96.5%), enrofloxacin (99.74%), ciprofloxacin (98.0%), sulfadimidine (99.6%), sulfamethoxazole (99.7%), sulfadiazine (99.8%), sulfachloropyridazine (99.1%) and sulfadimethoxine (99.7%) were obtained from Dr Ehrenstorfer GmbH (Augsburg, Germany). The final complement, which was aflatoxin M1 (10 µg/mL), ochratoxin A (≥98.0%) and zearalenone (99.0%) were procured from Trylogy Analytical Laboratory, Inc. (Washington, MO, USA).

Isotopically labelled internal standards. Clenbuterol-d6 HCl (98.0%), brombuterol-d9 HCl (98.0%), mabuterol-d9 HCl (98.0%), clenpenterol-d5 HCl (98.0%) and cimbuterol-d9 (98.0%) were purchased from Witega; isoxsuprine-d5 hemifumarate (≥98.0%) and ractopamine-d6 HCl (≥98.0%) were obtained from the European Reference Laboratory

at RIKILT (now Wageningen Food Safety Research, the Netherlands); 19-nortestosterone 17β was sourced from the RIVM National Institute for Public Health and the Environment (Bilthoven, the Netherlands); salbutamol-d9 (≥98.0%) was ordered from Dr Ehrenstorfer GmbH (Augsburg, Germany); zilpaterol–d7 (≥98.0%) and β-zearalenol-d4 (≥98.0%) were products of Toronto Research Chemicals Inc. (Toronto, Canada) and terbutaline-d9 acetate hemihydrate (99.3%), flunixin-d3 (100.0%), penicillin G-d7 N-ethyl-piperidinium (98.1%) salt, atrazine-d5 (99.7%), chlorpyrifos-d10 (100%) and carbofuran-d3 (99.3%) were obtained from Sigma-Aldrich.

Preparation of stock, intermediate and working standard solutions. Individual standards and internal standard stock solutions with concentrations from 0.5 to 1.0 mg/mL were prepared in methanol. Mixed working solutions from standards and internal standards with a concentration of 10 μg/mL were likewise prepared in methanol. Matrix-matched calibration curves were plotted from blank milk sample analysis. Before adding the standards, the blank milk samples were homogenised for 3 min on a rotary shaker. The concentration of the calibration curve is given in Supplementary Table 3. Isotopically labelled internal standards were used for the monitoring of the distribution of analytes in the extraction step and for the quantification.

Sample preparation. A total of 120 raw milk samples were collected from different dairy farms in North Macedonia. The collected samples were transported directly to the laboratory at 4°C and all samples were stored at 4°C until analysis.

In the first step, 30 g of milk sample was homogenised for 3 min on the rotary shaker. After homogenisation, 10 g of milk sample was fortified with the standards and internal standards. The concentrations of the added standards are given in Supplementary Table 5. The spiked sample was left to stand for 10 min. In the next step, 20 mL of extraction mixture consisting of acetonitrile:methanol:acetic acid 49.5:49.5:1, *v/v/v* was added and the samples were shaken for 1 min on a vortex mixer. The samples were shaken for 60 min with a rotary shaker and centrifuged at 8,000 rpm for 10 min at 0°C. The extraction step was repeated with an additional 20 mL of the extraction mixture. The supernatants from both extraction steps were combined and kept for 20 min at −80°C in a freezer. The solution was evaporated to near dryness at 35°C and the residue redissolved in 10 mL of methanol:water 10:90, v/v, after which it was shaken for approximately 1 min on the vortex mixer.

Extract cleanup. The Oasis HLB cartridges were activated and conditioned with 5 mL of methanol and 5 mL of water. The reconstituted extract (10 mL) was passed through the cartridges at one drop per s and the cartridge dried, washed with 5 mL of water and dried again. The residues were eluted with two eluent mixtures, first with 4 mL of eluent mixture I consisting of methanol:acetonitrile:ammonium hydroxide 47.5:47.5:5, *v/v/v* and then with 4 mL of eluent mixture II of methanol:dichlormethane 3:7, *v/v*. The solution was evaporated under nitrogen to near dryness at 35°C. The residue was reconstituted with 1 mL of mobile phase A:mobile phase B 95:5, *v/v* and finally the extracts were passed through a 0.45 μm membrane filter into 2 mL autosampler vials.

LC–MS/MS analysis. The analysis was carried out with an LC-MS/MS platform (Waters, Milford, MA, USA) equipped with a binary pump, vacuum degasser, thermostatted autosampler, thermostatted column manager and triple quadrupole detector. A Kinetex C18 column (50 × 2.1 mm, 2.6 μm, Phenomenex, Torrance, CA, USA) was used for the chromatographic separation. MassLynx software version 4.1 (Waters) was used for instrument control, data acquisition and calculation of results.

The flow rate for the mobile phase was 0.2 mL/min, the column temperature was 40°C, and the elution programme was as follows: 0–1 min 95–80% A; 1–4 min 80–60% A; 4–8 min 60–0% A; 8–10 min 0% A; 10–10.3 min 0–95% A and 10.3–12 min 95% A. Mobile phase A contained water with 5 mM ammonium acetate, 0.01% formic acid and 0.01% trichloroacetic acid, and mobile phase B contained methanol with 0.1% formic acid. The injection volume was 5 μL and the optimal MS/MS parameters were capillary voltage of 3.0 kV, source temperature of 150°C, desolvation temperature of 400°C, cone gas at 100 L/h and desolvation gas at 300 L/h. Electrospray positive and negative ionisation modes (ESI+ and ESI−) were used for the acquisition of the MS/MS parameters of the target compound and two multiple reaction monitoring (MRM) transitions were chosen.

Method validation. The method in this study was validated according to the criteria prescribed in European Commission Decision 2002/657 and the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use Q2 (R1) guidelines (2, 9). The validation parameters of linearity, accuracy, precision, LOD, LOQ, CCα and CCβ were evaluated. Matrix-matched calibration was used to assess the linearity. Certified reference materials not having been available, a recovery study was conducted, which was used for evaluation of the reliability of the results.

Results

LC–MS/MS optimisation. One precursor ion and two transitions were selected for the analysed compounds. The polarity, precursor ion, product ions, collision energy, cone voltage and retention time results for each compound are given in Supplementary Table 1. The optimal dwell time for a suitable signal-to-noise ratio and a good peak shape was 0.025 s.

Table 1. The mobile phases studied

No.	Mobile phase A	Mobile phase B
1	Water with 5 mM ammonium acetate and 0.1% formic acid	Acetonitrile with 0.1% formic acid
2	Water with 5 mM ammonium acetate and 0.1% formic acid	Acetonitrile:methanol (50/50; v/v) with 0.1% formic acid
3	Water with 5 mM ammonium acetate and 0.1% formic acid	Methanol with 0.1% formic acid
4	Water with 5 mM ammonium acetate and 0.01% formic acid	Methanol with 0.1% formic acid
5	Water with 5 mM ammonium acetate, 0.1% formic acid and 0.01% trichloroacetic acid (TCA)	Methanol with 0.1% formic acid
6	Water with 5 mM ammonium acetate, 0.01% formic acid and 0.01% TCA	Methanol with 0.1% formic acid

TCA – trichloroacetic acid

The LC conditions such as the mobile phase composition were investigated after optimisation of the MS parameters. Different mobile phases were studied (Table 1) because of the considerable number of components included in this method and differences in their chemical structure. When using mobile phase A and B pair 1 as shown in Table 1, with A of water with 5 mM ammonium acetate and 0.1% formic acid and B of acetonitrile with 0.1% formic acid, zeranol, taleranol, ampicillin, benzylpenicillin, cloxacillin, parathion, atrazine, permethrin, cypermethrin, chlorpyrifos, fenvalerate, fenthion, amitraz and bifenthrin were not detected. Using mobile phase pair 2, detection of the compounds from groups A3 (anabolic hormones) and A5 (β-agonists), as well as β-lactams, lincomycin, tylosin and cefapirin failed, and with mobile phase pairs 3 and 4 benzylpenicillin, cefapirin, permethrin, oxytetracycline, tetracycline and phosmet were undetectable. From the chromatogram peaks for 19-nortestosterone, methacriphos and cefalexin readable in Supplementary Figs 1, 2 and 3, it can be concluded that the peaks were splitting. Moreover, with mobile phase pair 3, only one product ion was detected among the malathion, parathion and chlorpyriphos compounds. The results are shown in Supplementary Figs 4, 5 and 6. Taleranol, cefalexin, phosmet and amitraz were elusive to detection with mobile phase pair 5. Also, Supplementary Figs 7, 8, 9 and 10 present the detection of a mere one product ion for ampicillin, benzylpenicillin, cloxacillin and malathion. The optimal mobile phase was water with 5 mM ammonium acetate, 0.01% formic acid and 0.01% trichloroacetic acid (TCA) as mobile phase A and methanol with 0.1% formic acid as mobile phase B.

Optimisation of the sample preparation. Three solvents were investigated for the extraction of 69 compounds from bovine milk and acetic acid was included in all of them. Acetonitrile:methanol:acetic acid 49.5:49.5:1, v/v/v, acetonitrile:ethyl acetate:acetic acid 49.5:49.5:1, v/v/v and methanol:ethylacetate:acetic acid 49.5:49.5:1, v/v/v were evaluated by using the spiked samples at three concentration levels. The standards and internal standards were added to the bovine milk samples before sample work-up. The best recoveries (70.83% to 109%) were achieved with the first solvent, while the recoveries with the second and third were from 61.45% to 105.32% and 60.08% to 107.32%, respectively. The results are provided in Supplementary Table 2.

Single extraction, double extraction and triple extraction with 20 mL acetonitrile:methanol:acetic acid 49.5:49.5:1, *v/v/v* in each step were also compared. Double extraction delivered higher recoveries than single extraction, while triple extraction did not significantly improve the recoveries. Consequently, double extraction with 20 mL of the stated solvent was chosen.

Validation of the results' linearity. Matrix-matched calibration curves were prepared for the evaluation of the method's linearity. The range of the calibration curve and coefficient of correlation (R^2) for each compound are shown in Supplementary Table 3.

Performance parameters LOD, LOQ, CCα and CCβ. The LOD and LOQ were calculated from the lowest levels of the standards which were used for the calibration curve as follows: the LOD was calculated as the mean value (n = 6) from the lowest standard plus 3.3 times the calculated standard deviation, while the LOQ was the mean value (n = 6) from the lowest standard plus 10 times the calculated standard deviation. The CCα and CCβ were calculated according to the criteria in European Commission Decision 2002/657/EC (2). The range of the LODs was 0.036 μg/kg–47.94 μg/kg, the low value being for clenbuterol and the high value for lincomycin, while the range of the LOQs was 0.053 μg/ kg–59.43 μg/kg, the low value again being for clenbuterol and the high being for bifenthrin. The CCα values were in the range 0.062 μg/kg to 211.32 μg/kg, and the CCβ ranged from 0.080 μg/kg to 233.71 μg/kg, the low and high values for both parameters being for the same standards as for the LOQ. The results for the LODs, LOQ, CCα and CCβ are summarised in Supplementary Table 4.

Accuracy and precision. The results are shown in Supplementary Table 5. They ranged from 70.83% for benzylpenicillin (spiked at a concentration of 2 μg/L) to

109.00% for dimethoate (spiked at a concentration of 15 µg/L). The precision of the method was expressed through the coefficient of variation (CV, %). The CV for repeatability (intra-day precision) was from 2.41% for cefapirin (spiked at 90 µg/L) to 22.29% for zilpaterol (spiked at 15 µg/L), and the CV for reproducibility (inter-day precision) was from 3.48%, again for cefapirin (spiked at 90 µg/L), to 23.91%, also for zilpaterol (spiked at 15 µg/L). The chromatograms of the milk samples spiked with standards at concentration level two from Supplementary Table 5 are shown in Figs 1 a, b, c and d.

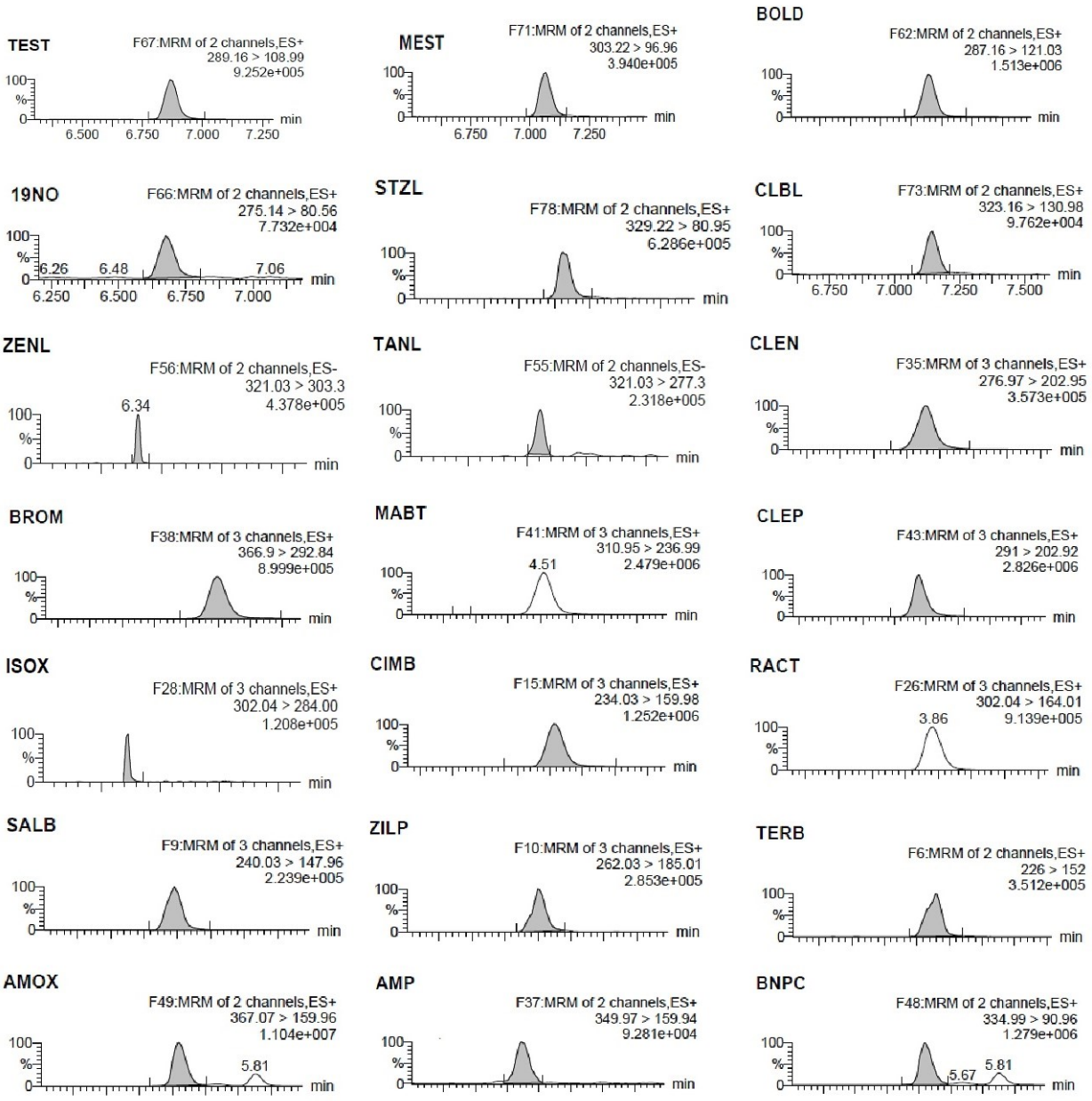

Fig. 1a. The chromatograms of spiked milk samples at concentration level 2 from Supplementary Table 5
TEST – testosterone; MEST – methyltestosterone; BOLD – boldenone; 19NO – 19-nortestosterone; STZL – stanozolol; CLBL – clostebol; ZENL – zeranol; TANL – taleranol; CLEN – clenbuterol; BROM – brombuterol; MABT – mabuterol; CLEP – clenpenterol; ISOX – isoxsuprine; CIMB – cimbuterol; RACT – ractopamine; SALB – salbutamol; ZILP – zilpaterol; TERB – terbutaline hemisulphate; AMOX – amoxicillin; AMP – ampicillin; BNPC – benzylpenicillin

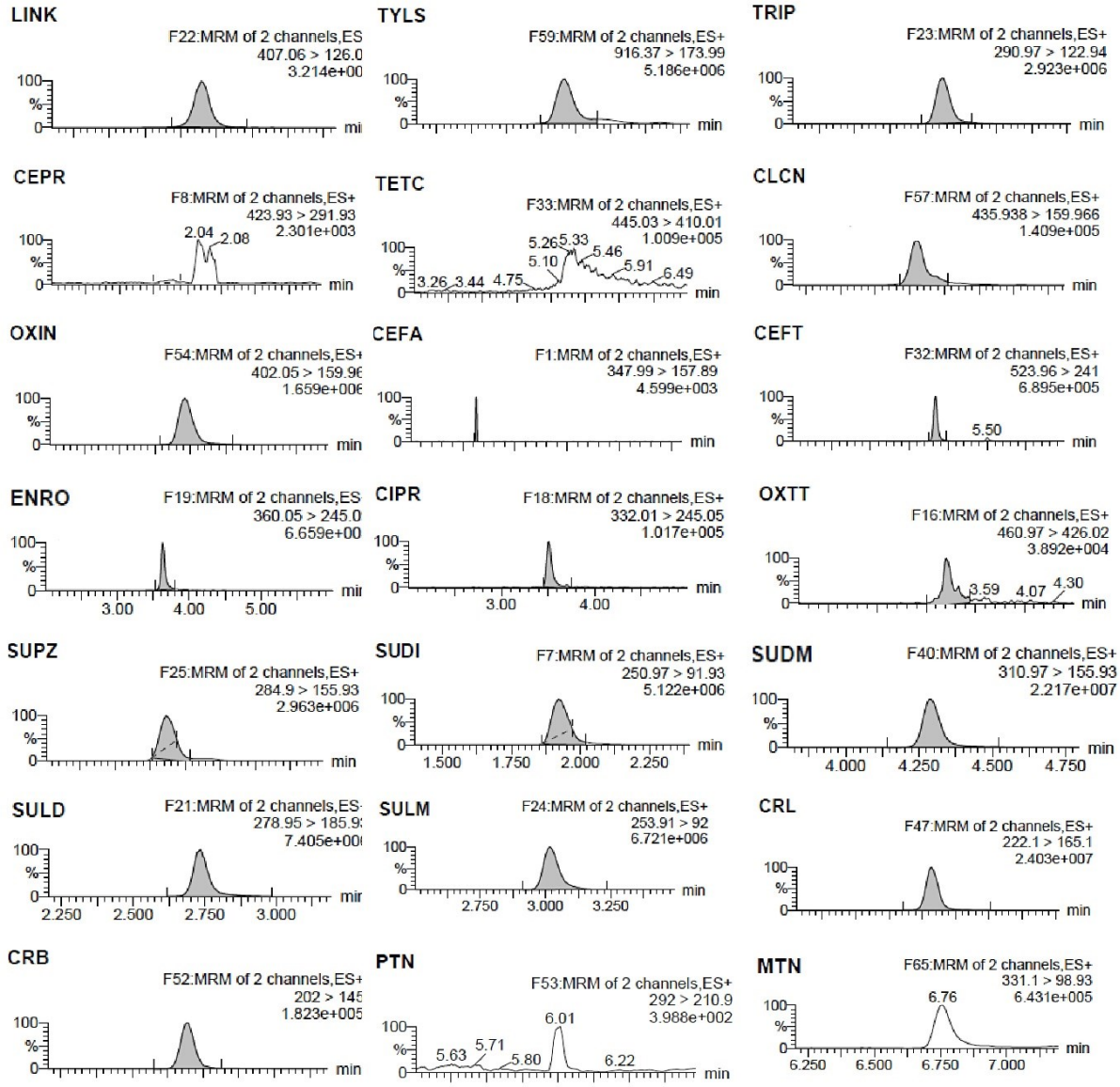

Fig. 1b. The chromatograms of spiked milk samples at concentration level 2 from Supplementary Table 5
LINK – lincomycin; TYLS – tylosine; TRIP – trimethoprim; CEPR – cefapirin; TETC – tetracycline; CLCN – cloxacillin; OXIN – oxacillin; CEFA – cefalexin; CEFT – ceftiofur; ENRO – enrofloxacin; CIPR – ciprofloxacin; OXTT – oxytetracycline; SUPZ – sulfachloropyridazine; SUDI – sulfadiazine; SUDM – sulfadimethoxine; SULD – sulfadimidine; SULM – sulfamethoxazole; CRL – carbaryl; CRB – carbofuran; PTN – parathion; MTN – malathion

Discussion

To optimise the MS/MS parameters and select the appropriate diagnostic ions, direct infusion of the single-analyte standards with a concentration of 1.0 µg/mL into the ESI source was used. Each compound gave a spectrum in scan mode for the mass range m/z 50–1000. For each compound, the precursor ion and two MRM transitions were selected, and consequently the product ions with the highest intensity were chosen as ions for quantification of the compounds (product ions for quantification for all compounds are shown in Supplementary Table 1). Sixty-six compounds (95.65%) were detected using ESI in the positive mode, and the remaining three (4.35%) were detected using ESI in the negative mode.

The most common mobile phases for antimicrobial residue studies using LC-MS/MS are methanol and/or acetonitrile–water, and formic acid is often added at low levels to facilitate ionisation by ensuring the analyte is more basic than the solvent. In this study, the optimal mobile phase A was water with 5 mM ammonium acetate, 0.01% formic acid and 0.01% TCA and the optimal mobile phase B was methanol with 0.01% formic acid because of the improved separation, good peak shape, and high signal intensity for the investigated compounds. Therefore, a gradient elution of water with 5 mM ammonium

acetate, 0.01% formic acid and 0.01% TCA provided the best peak symmetry and resolution of the target compounds. The study showed that the use of other mobile phases led to poor separation, poor peak shape, low signal intensity or, in some cases, failure to detect certain compounds. In summary, the optimum chromatographic conditions used were 0.2 mL/min flow rate, full loop injection volume of 5 µL and a Kinetex C18 column of 50 × 2.1 mm and 2.6 µm. Separation was achieved by using 0–1 min 95–80% A; 1–4 min 80–60% A; 4–8 min 60–0% A; 8–10 min 0% A; 10–10.3 min 0–95% A and 10.3–12 min 95% A as the gradient mobile phase. The column temperature was 40°C. The total time required for the chromatographic analysis was 12 min, which was shorter than the time of 20–40 min occupied by equivalent analysis in previous work (24, 28).

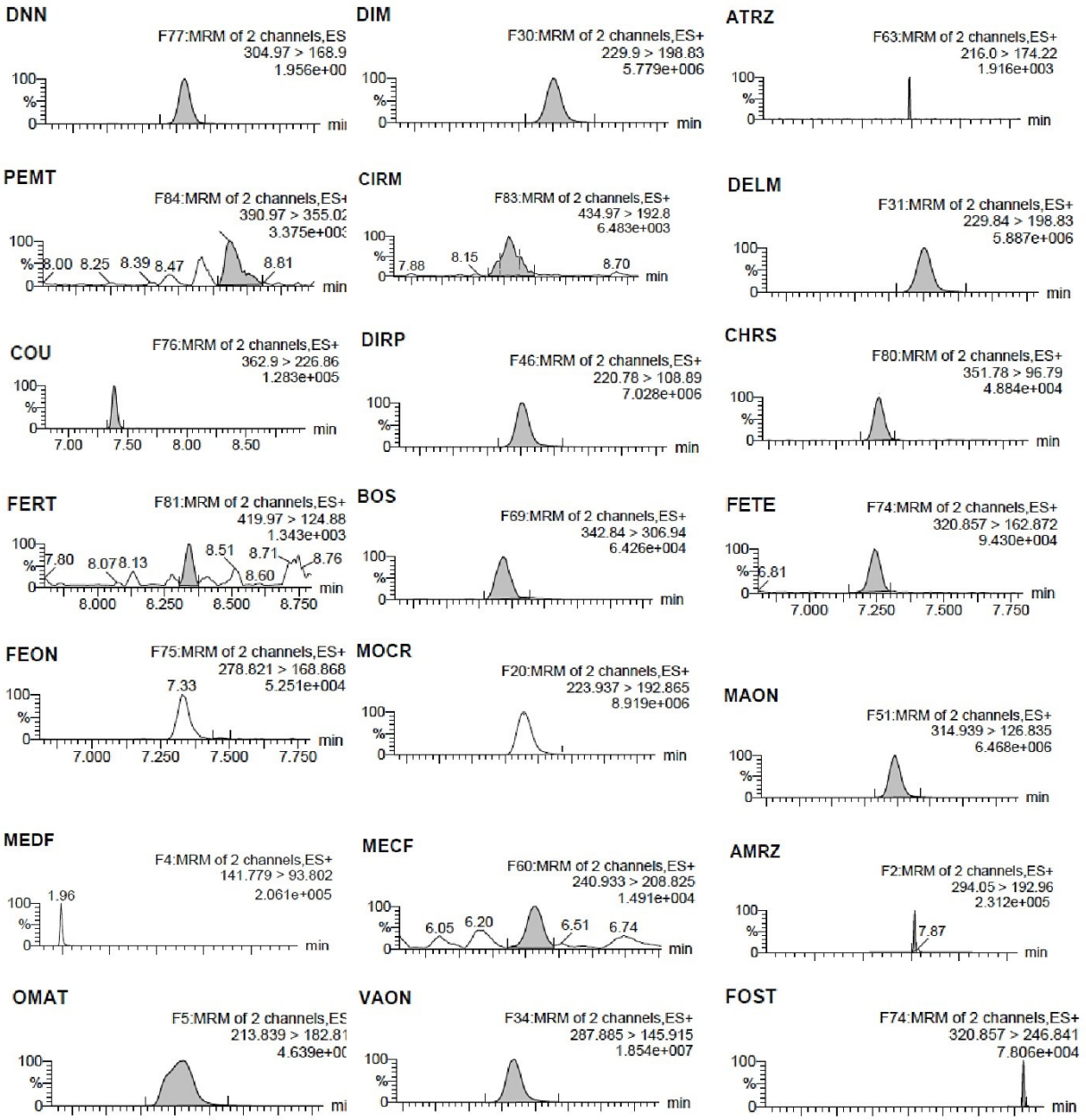

Fig. 1c. The chromatograms of spiked milk samples at concentration level 2 from Supplementary Table 5
DNN – diazinon; DIM – dimethoate; ATRZ – atrazine; PEMT – permethrin; CIRM – cypermethrin; DELM – deltamethrin; COU – coumaphos; DIRP – dichlorvos; CHRS – chlorpyrifos; FERT – fenvalerate; BOS – boscalid; FETE – phenthoate; FEON – fenthion; MOCR – monocrotophos; MAON – malaoxon; MEDF – methamidophos; MECF – methacrifos; AMRZ – amitraz; OMAT – omethoate; VAON – vamidothion; FOST – phosmet

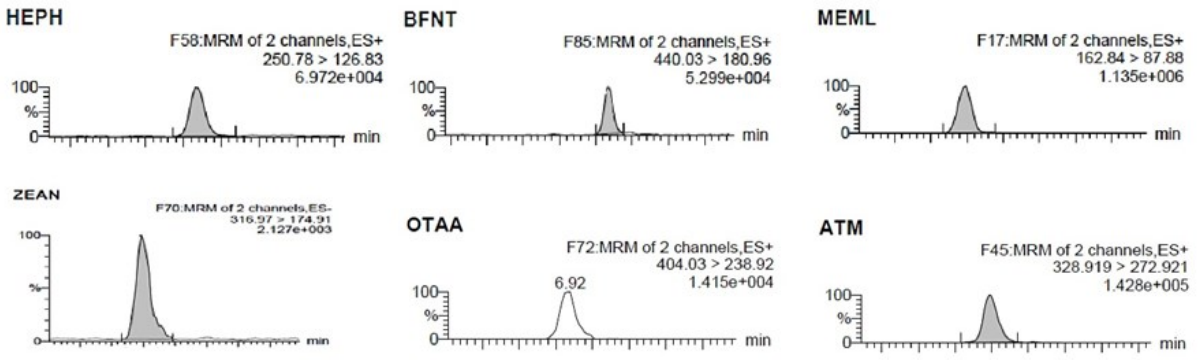

Fig. 1d. The chromatograms of spiked milk samples at concentration level 2 from Supplementary Table 5
HEPH – heptenophos; BFNT – bifenthrin; MEML – methomyl; ZEAN – zearalenone; OTAA – ochratoxin; ATM – aflatoxin

The sample preparation procedure is the critical step in the application of the multi-class and multi-residue method. The composition of milk most often leads to difficulties in the analytical determination of antimicrobials. The high content of carbohydrates/lactose, fat and protein can often interfere with the analytical method (18, 21, 24). The most common solvent used for extraction of residues and contaminants from milk is acetonitrile, which is better than other solvents such as acetone, ethanol and methanol in matrix precipitation. A negative effect of the use of pure acetonitrile in extraction is that phases may be binary between an aqueous and an acetonitrile phase (5, 17, 25). Zhan *et al.* (26) reported that an acetonitrile : alcohol 5 : 1, v/v combination provides good precipitation and higher analyte response intensity than extraction with the individual use of alcohol, acetone or methanol. A further reason for a solvent combination is that many residues and contaminants differ in physicochemical properties, such as polarities, pKa values and stability; therefore, use of a combination of solvents is needed for better extraction. Xie *et al.* (23) investigated the influence of pH on recoveries with three extraction protocols, namely acetonitrile : ethyl acetate : acetic acid 49.5 : 49.5 : 1, *v/v/v*, acetonitrile : ethyl acetate : ammonium hydroxide 49.5 : 49.5 : 1, *v/v/v* and acetonitrile : ethyl acetate 1 : 1, *v/v*, and reported that the best recoveries were achieved with the first protocol, when the acetic acid was used with acetonitrile and ethyl acetate. That study included 40 compounds belonging to 17 different classes in milk, yogurt, milk powder and cheese samples. Sulfonamides and lactones were not included, and only two pesticides were in the experiment design. In the present study, sulfonamides, lactones and several pesticides were included and the method was optimised for 69 residues and contaminants in bovine milk.

The results were mostly very satisfactory and met the requirements listed in European Commission Regulation 2002/657/EC for linearity, LOD, LOQ, repeatability, reproducibility, recovery, CCβ and CCα. The R^2 of the calibration curve standards was ≥0.995. This value was acceptable for each target compound. The LOD and LOQ values for all compounds which have a maximum residue limit (MRL) set for their residue in milk were lower than that MRL value (2, 3, 6, 7, 8). The results for LOD, LOQ, CCα and CCβ also showed good sensitivity and agreed with the requirements of Regulation 2002/657/EC (2). The accuracy of the method was expressed by recovery percentage. Recovery and intra-day precision (repeatability) were evaluated at three different concentration levels. For this purpose, the milk samples were spiked with a mixed standard solution and had the standards and internal standards added before sample work-up. For each concentration level, six replicates were prepared. The inter-day precision (reproducibility) was evaluated as repeatability, but the spiked samples were prepared and analysed on three consecutive days, also in six replicates for each concentration level. The method thus demonstrated good accuracy and precision and was in compliance with the criteria described in the relevant Commission Regulation (2).

For assessment of the method's applicability, it was applied to the analysis of samples of commercial milk. The results showed the presence of residues from antibiotics in five bovine milk samples: sulfadimethoxine in two samples and enrofloxacin, tetracycline and oxytetracycline each in one with concentrations of 27.4 µg/L, 18.2 µg/L, 25.7 µg/L, 30.1 µg/L and 41.3 µg/L, respectively. The concentration of residues in bovine milk samples was lower than the EU MRL.

In conclusion, this paper presents a multi-class method proven for bovine milk samples for the extraction, clean-up, identification and quantification of 69 residues of veterinary drugs and other contaminants, including anabolic hormones, lactones, β-agonists, antimicrobials, pesticides and mycotoxins. Isotopically labelled internal standards were used for the monitoring of the distribution of residues and contaminants in the extraction procedure and for quantification. Different

sample preparation techniques are suitable for different animal food matrices, each with its own advantages and disadvantages. The mobile phase composition and sample preparation for multi-class analysis in bovine milk were optimised. Good validation results were obtained by taking into account the difficulties of a multi-class, multi-residue method for the complicated matrix adopted for this research. The method could be used in routine analysis of bovine milk samples for simultaneous detection of the veterinary drug residues, mycotoxins and pesticides with which this research concerned itself. Further work involving this method will likely expand its applications to different food matrices such as animal feed and other food derived from animals such as organs, honey and eggs.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: None required.

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