

Committee on Drugs and Related Topics

Drug Residues in Animal Tissues

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Summary

A report on the collaborative study on the determination of clopidol residues in chicken muscle is under review by the Committee. Out of 18 participating laboratories, 16 reported results without significant deviations from the method. Results from the 2 remaining laboratories were excluded due to deviations from the protocol. Extracts are cleaned up on alumina and anion exchange solid-phase extraction cartridges, then analyzed for clopidol using reversed-phase liquid chromatography with UV detection at 270 nm. Recoveries were >80% over the analytical range (0.10–5.00 mg/kg) and accuracy and precision were in conformity with required performance standards for the concentration range. No other studies have been approved or completed since the last report.

Significant developments in the application of optical immunobiosensor technology have been reported in the past year and this technology shows promise for application in many aspects of the detection of veterinary drug residues. The determination of the bronchodilator clenbuterol, banned in many countries from use in food animals due to toxicity concerns and its unapproved use as a repartitioning agent, was accomplished using a monoclonal antibody immobilized on a sensor chip (1). Urine samples were extracted with *tert*-butyl ether, then applied directly to the biosensor chip for analysis. Up to 88 samples could be analyzed in a working day, with each cycle of the biosensor requiring approximately 7 min for sample application, analysis and flushing prior to analysis of the next sample. Analyses were conducted with reference to a calibration curve prepared in buffer. Recoveries were in the range of 95%, with a detection limit of 0.00027 mg/L. Results were comparable with those obtained using enzyme immunoassay, radioimmunoassay and GC-MS. The biosensor showed cross-reactivity with 7 other β -agonist compounds related to clenbuterol and was capable of detecting them at concentrations <0.001 mg/L in bovine urine. This technology has also been applied to the detection of streptomycin and dihydrostreptomycin residues in milk, honey, and pig kidney and muscle (2). The method was rapid and rugged, allowing direct analysis of whole milk, while honey samples required a dilution with buffer and tissue samples homogenized in buffer, then centrifuged to remove solids. Limits of detection ranged from 0.015 mg/kg in honey to 0.070 mg/kg for muscle,

with relative standard deviations of 2.8–11% across the range of matrixes at concentrations from 0.020–0.500 mg/kg. The advantages of high test sensitivity and specificity, combined with minimal sample preparation, make this method an attractive approach for rapid screening of samples for targeted residues. The preparation of sensor chips and factors to consider in optimizing performance, using clenbuterol, ethinylestradiol, and trenbolone as sample analytes, was the subject of a recent publication (3).

Interest continued in the analysis of β -agonist drugs, both as a result of continued concern about nonapproved use of some of these compounds, such as clenbuterol and salbutamol, and with the registration for use in some countries of ractopamine and zilpaterol as production aids. The use of immunofiltration as a cleanup for the immunochemical detection of β -agonists was studied as a means to improve screening methods (4). Free antisalbutamol polyclonal antibodies (0.25 mL) were mixed with 0.25 mL test portion of urine (pH 7 ± 1) in an ultrafiltration device with a 30 kDa cut-off. After mixing on a Vortex mixer and centrifugation at 5000 *g* for 15 min, 0.2 mL PBS was added to the device, which was then centrifuged for an additional 15 min. The filtrate was removed and 0.3 mL methanol–0.1 mol/L acetic acid (1 + 1, v/v) was added to the retentate to free the bound β -agonists. After centrifugation at 5000 *g* for 45 min, this filtrate was evaporated to dryness at 50°C under a stream of nitrogen and the residue was taken up in 0.5 mL PBS. A 0.050 mL aliquot was then analyzed by an ELISA for β -agonists. The combination of the immunodiffusion cleanup with the ELISA was found effective for detection of 11 β -agonists that were tested, providing a 30-fold increase in test sensitivity over direct analysis of urine by the ELISA. The limit of detection for the combined techniques was 0.00014 mg/L salbutamol equivalents. Performance of the test was successfully demonstrated on known incurred samples which had previously been analyzed by GC/MS. In another study, the application of an ELISA using a monoclonal antibody for the detection of ractopamine residues was reported, with the ELISA results being compared to liquid chromatographic results on incurred samples from cattle and sheep (5). Good correlation of ELISA and LC results was obtained for beef liver and for urine samples which had been treated with hydrolytic enzymes (glucuronidase/aryl-sulfatase), but ELISA gave higher results than LC for sheep urine which had not been hydrolyzed. Less than 0.001 mg/kg free ractopamine was detected in tissues after 7 days withdrawal, while residues in urine declined rapidly during the 7 day withdrawal period to concentrations below the limits of quantitation for parent compound of both the ELISA (0.0015 mg/L) and the LC (0.005 mg/L) methods.

Potential contamination problems associated with testing of hair as an indicator of treatment with β -agonists was also investigated (6). Sampling of hair as a means of detection of illegal use of these drugs is an attractive option, as sample collection on live animals is considered simpler and less invasive than sampling for blood or urine. However, there have been concerns that hair could be contaminated by external contact, leading to positive results for animals which had not been treated with these drugs. It was demonstrated that bovine hair when exposed to aqueous solutions of β -agonists incorporates residues of the drug into the hair and that the residues cannot be removed by a variety of washing treatments, including detergents and organic solvents. Clenbuterol was extracted from hair using 0.1 mol/L hydrochloric acid and it was found that the extraction kinetics differ for residues resulting from external exposure when compared to incurred residues. Further study is planned to investigate the differences which may be seen when hair is tested at a prolonged period after external contamination, as opposed to sampling shortly after the contamination, as was done in the present study.

An investigation was also reported on the detection of 17 α -ethinylestradiol residues in the hair of cattle which received the drug by intramuscular injection at 2 mg/kg bodyweight (7). After washing 3 times with a detergent solution, followed by a rinse with distilled water, 0.500 g test portions of hair were digested at 100°C for 10 min in 2.5 mL sodium hydroxide. The digestate was adjusted to pH 8.4 by addition of 0.2 mol/L acetate buffer in a 500 mL beaker, then a dialysis tube (molecular cut-off 10 000 Da) containing 25 mL dichloromethane was placed in the beaker. The beaker contents were stirred at 150 rpm for 4 h at 37°C in an incubator-shaker, then the dialysis tube contents were decanted into a glass tube and dried under a nitrogen stream. The residue was derivatized to form TMS derivatives and a 0.002 mL aliquot was injected into the gas chromatograph, equipped with a capillary column containing a cross-linked 5% phenyl-methylpolysilane coating. The ion trap detector was operated in electron impact mode to produce the m/z 425 parent ions and product ions at m/z 397 and 407. Recoveries of 74–94% were obtained at concentrations from 0.004–0.020 mg/kg, with relative standard deviation <20%. The limit of detection calculated was 0.0005 mg/kg. Residues of 17 α -ethinylestradiol (approximately 0.002–0.020 mg/kg) were detected in hair samples collected from the 5 experimentally treated animals beginning at 7 days following exposure and continuing to 98 days post-treatment, when final samples were collected.

Methodology for the determination of ractopamine residues in pork liver, kidney and muscle, tissues with enriched β -agonist receptors (lung, retina) and bovine urine has also been reported (8). A 15 g test portion of tissue (0.20–0.50 g for retina) is freeze-dried, ground, then mixed with 12 mL methanol and 15 mL 2 mol/L acetate buffer (pH 5.2) and stirred for 30 min. After centrifugation at 2000 g for 15 min, the supernatant is collected and the methanol is evaporated. A *helix* pomatia solution (0.40 mL) is added and the mixture is incubated at 60°C for 15 h. Subsequent cleanup was done using

2 solid-phase extraction cartridges and the dried final extract was taken up in 0.050 mL 0.5% aqueous acetic acid–methanol (95 + 5, v/v), with a 0.010 mL aliquot taken for LC/MS/MS analysis. For urine, a 10 mL test portion was mixed with 2 mL 2M acetate buffer (pH 5.2) and taken through the hydrolysis and cleanup steps. The MS–MS collision gas was argon and sample analysis was in the multiple reaction monitoring mode. The pseudo-molecular ion, m/z 302, was used to monitor for both ractopamine and the internal standard, isoxsuprine. Six diagnostic product ions were obtained for ractopamine, m/z 284, 164, 136, 121, 107, and 91. Validation was according to the draft EU criteria, beginning with the analysis of 20 blank pork tissue samples from different origins to demonstrate specificity. The blank samples were then pooled and fortified test portions were used to determine the linearity of the calibration curve at concentrations from 25–500 ng/kg. The decision limit ($CC\alpha$) was calculated to be 9 ng/kg, with a detection capability ($CC\beta$) of 28 ng/kg. $CC\alpha$ is the lowest concentration at which a method can detect the presence of an analyte with a statistical certainty of 1- α , in this case calculated at 99% certainty. $CC\beta$ is defined as the lowest concentration at which a method can detect truly contaminated samples with a statistical certainty of 1- β . Again, the authors calculated using a 99% confidence level. The method as validated was considered to meet the draft EU guidelines for validation of methods for residues of veterinary drugs and provides an example of the experimental design and calculations required. Further detail on this approach to method validation, with definitions and examples, is contained in 2 recent publications (9, 10). Analysts should be aware of these requirements and should consider them carefully in designing and conducting validation experiments within their laboratories. Further examples of methods validated using these draft guidelines are referenced throughout this report.

A method for the determination of residues of the anticoccidial drug pyrimethamine in animal tissues and eggs using liquid chromatography with fluorescent detection had a detection limit of 0.010 mg/kg and recoveries >60% across the range of matrixes tested at concentrations of 0.10 to 1.0 mg/kg (11). Samples were extracted in acetonitrile, centrifuged, defatted with hexane, then evaporated to dryness. The residue was dissolved in pH 10 buffer and extracted with dichloromethane, again taken to dryness and then derivatized with chloroacetaldehyde to produce fluorescence with excitation at 300 nm and emission at 420 nm. No pyrimethamine residues were detected in any of the 70 samples tested, which included swine muscle, chicken muscle, and liver and eggs.

A method for clopidol residues in chicken tissues and eggs, which was the subject of a collaborative study, was reported, along with additional information on the distribution and depletion of clopidol residues in chickens and the application of the method to over 7000 samples in a residue control program (12–14). Following extraction with acetonitrile, extracts are passed through alumina and anion exchange solid-phase extraction cartridges to remove co-extractives, then analyzed by reversed-phase liquid chromatography with UV detection at 270 nm. The reported limit of detection for the method was

0.005 mg/kg, with recoveries >85% from fortified chicken muscle, kidney, and eggs at concentrations from 0.01–2.0 mg/kg. Highest clopidol residues at 8 h following treatment were in liver (4.6 mg/kg), followed by kidney (3.6 mg/kg), and muscle (1.5–1.7 mg/kg). Residues deplete rapidly and were not detectable at 7 days withdrawal.

A multiresidue determinative method for residues of the 5 anticoccidial drugs (nicarbazin, and the ionophores lasalocid, monensin, salinomycin, and narasin) in liver and eggs using LC–MS/MS provided recoveries from fortified poultry livers of 92–118%; while recoveries from eggs were 86–110% (15). All analytes were quantifiable at 0.0025 mg/kg and the method has the advantage of a high sample throughput of 40 samples/analyst/day. Methodology was also reported for the determination of the ionophore polyether antibiotic lasalocid, used as a feed additive for the prevention of coccidiosis in chickens, in a range of processed foods, including baby food, sausages, and meat pastries (16). The method was an adaptation of a method previously developed for the analysis of raw animal tissues (17). Samples are homogenized in acetonitrile in the presence of anhydrous sodium sulfate, cleaned up on silica solid-phase extraction cartridges, followed by an NH₂ cartridge, then analyzed on a polymeric liquid chromatographic column with fluorescence detection (excitation 310 nm, emission 420 nm). The method was validated at concentrations from 0.01–0.04 mg/kg in products which included egg- and chicken-based baby foods, chicken liver, meat pies, pâtés, and pork sausages, with recoveries of 78–96% and RSDs of 5–14%. A similar approach was described for the analysis of residues of the ionophore polyethers narasin, monensin, and salinomycin in chicken livers and eggs, where LC–MS/MS methodology was used to screen for residues of these compounds in a national survey and also to provide confirmatory capability for positive findings (18). Following an automated solid-phase extraction cleanup, samples were analyzed with a run-time of 4 min per sample, with the added advantage that the low limits of detection (0.000026 mg/kg for narasin) enabled sample pooling with detection of any potential violations of the Swedish maximum residue limits. Recoveries for narasin were 94–108%, with relative standard deviations of 4–10% for narasin in eggs fortified at 0.5–20 ng/g.

A method using LC–MS/MS analysis for the confirmation of residues of nicarbazin in chicken liver and eggs was also reported, with validation according to requirements of a draft revised criteria document for validation of veterinary drug residue methods discussed above, which is currently under consideration by authorities within the European Union (19). The method provides quantitative confirmation of the marker residue, 4,4'-dinitrocarbanalide (DNC), and provides a discussion of the practical application of the calculation of the "decision limit," or CC α , and detection capability, or CC β , as required in the revised criteria document. The analysis is based on the monitoring of the [M–H][–] ion at m/z 301 and the transition ions at m/z 137 and 107 and uses d₈-DNC as an internal standard. Method performance was demonstrated at

concentrations from 0.010–0.100 mg/kg for residues in eggs and from 0.100–0.300 mg/kg for residues in liver.

A method was developed for the determination of residues of the anthelmintic drug levamisole in muscle, liver, kidney and fat of sheep, pigs, and poultry (20). A 5 g test portion of tissue was mixed with 1 g sodium chloride, 1 g sodium bicarbonate, and 10 mL chloroform, then homogenized for 5 min at 20 500 rpm. The homogenate was centrifuged for 15 min at 3000 g, the chloroform layer was decanted, and the extraction was repeated with an additional 20 mL chloroform. The chloroform extracts were combined, evaporated to dryness, and the residue was dissolved in 5 mL chloroform–cyclohexane (1 + 3, v/v). The extracts were loaded on a silica solid-phase extraction cartridge that had been previously conditioned with 5 mL chloroform–cyclohexane (1 + 3, v/v), washed with 5 mL of this mixture, then the levamisole was eluted with 2 mL methanol and evaporated to dryness. The residue was dissolved in 0.5 mL mobile phase for analysis. Liquid chromatography was on a reversed-phase column using a mobile phase consisting of methanol–0.05 mol/L ammonium acetate buffer (55 + 45, v/v) at a flow rate of 1 mL/min, with UV detection at 220 nm. Determination was with reference to an internal standard, thiabendazole. Recoveries were >80% in all tissues for the 3 species, with relative standard deviations <5%. The limit of quantitation was reported as 0.004 mg/kg.

Interest in the analysis of compounds of the avermectin/milbemycin group continued. A method for the simultaneous determination of residues of emamectin and ivermectin in muscle tissue from Atlantic salmon used reversed-phase liquid chromatography with fluorescence detection (excitation 365 nm, emission 470 nm), with detection limits of 0.0005 mg/kg (21). Samples are extracted with acetonitrile, cleaned up on a C18 solid-phase extraction cartridge, then derivatized with 1-methylimidazole and trifluoroacetic anhydride. The limit of quantitation is 1.5 ng/g, with recoveries of 96 ± 9% for emamectin and 86 ± 6% for ivermectin. Methodology for the determination of abamectin and ivermectin in cattle plasma also used an initial acetonitrile extraction, but cleanup was on an alumina solid-phase extraction cartridge (22). The same derivatization was used, with analysis by reversed-phase liquid chromatography and fluorescence detection (excitation 365 nm, emission 475 nm). Recoveries were 91–100% for ivermectin and 87–99% for abamectin, with a reported detection limit of 0.00002 mg/L in 1.0 mL samples. Residues of ivermectin and doramectin in pig liver were determined after initial extraction with acetonitrile, centrifugation and derivatization with 1-methylimidazole (23). As in the previously described methods, analysis was by reversed-phase liquid chromatography with fluorescence detection. Recoveries were 75% for ivermectin and 70% for doramectin, with a limit of detection of 0.8 ng/g for each compound.

Similar methodology was reported for the determination of eprinomectin in milk, based on detection of the marker residue eprinomectin B_{1a} (24). After initial extraction of a 2 mL test portion with 3 mL acetonitrile, followed by mixing on a Vortex mixer, then centrifugation, a 1 mL aliquot of extract was

dried and taken up in a 30% solution of 1-methylimidazole in acetonitrile. Within 5 min of injection, the autosampler was programmed to add 0.3 mL 30% trifluoroacetic acid in acetonitrile to the sample vial, with the subsequent reaction to form the analyte derivative with the 1-methylimidazole occurring within 30 s at room temperature. A related experimental compound (designated MK-324) was added to each sample prior to extraction as an internal standard. Liquid chromatographic analysis was on a reversed-phase column under isocratic conditions at a column temperature of 35°C, using a mobile phase of methanol, acetonitrile, water, triethylamine, and *ortho*-phosphoric acid (25 + 68 + 7 + 0.2 + 0.2, v/v) at a flow rate of 1.0 mL/min. The fluorescent derivatives of eprinomectin and internal standard were detected with an excitation wavelength of 365 nm and an emission wavelength of 475 nm. A recovery of 94% was reported for eprinomectin B_{1a} from milk fortified at concentrations from 0.002–0.050 mg/L, with relative standard deviations <5% in the 2 laboratories which tested the method. The limit of quantitation was established at 0.002 mg/L.

Abamectin, doramectin, eprinomectin, ivermectin, and moxidectin residues were extracted from beef, pork, and sheep livers using supercritical carbon dioxide (25). A 2.5 g test portion was mixed with 4.0 g hydromatrix, then added to an extraction vessel containing 2.0 g basic alumina. Following equilibration of the sealed extraction vessel with pressurized CO₂, a volume equivalent to 50 L of expanded gas was passed through the vessel. The alumina was then removed from the depressurized vessel and transferred into an empty SPE cartridge and the analytes were eluted with 4 mL methanol–ethyl acetate (70 + 30, v/v) and collected in a silanized test tube, where the solvents were evaporated. The fluorescent derivatives were prepared by addition of 1-methylimidazole and trifluoroacetic acid solutions in acetonitrile, followed by glacial acetic acid, then incubated at 65°C for 30 min prior to analysis. Liquid chromatography was conducted under isocratic conditions on a reversed-phase column maintained at 30°C, using a mobile phase of methanol–acetonitrile–1% triethylamine/1% phosphoric acid in water (60 + 30 + 10, v/v/v). The limit of quantitation was established at 0.002 mg/kg, based on the lowest standard used in the calibration curve. Recoveries from beef liver (between assay) were 76–85% for all 5 compounds at 0.004 and 0.020 mg/kg fortification levels, with relative standard deviations 8.8–16.9%. Recoveries from pork liver and sheep liver were slightly lower (71–79%, with relative standard deviations 3.3–15.6%) at a fortification concentration of 0.010 mg/kg. Using equipment which allowed processing of 2 test portions at a time, a sample throughput of 10 samples per day was achieved.

Supercritical fluid extraction was also applied in the recovery of the acetyl gestagenic steroids (megestrol acetate, or MA; medoxyprogesterone acetate, or MPA; chlormadinone acetate, or CMA; and melengestrol acetate, or MGA) from kidney fat (26). As in the method described above, residues were extracted with supercritical carbon dioxide and trapped on alumina in the extraction vessel. A 2 g test portion of tissue is first blended with 2 g Extrelut[®], with addition of 0.5 mL of

water, then the mixture is placed in an extraction vessel. After insertion of a polypropylene frit over the test material, the extraction vessel is filled with 2–3 g alumina, sealed and the extraction proceeds with liquid carbon dioxide under static conditions for 5 min, then under dynamic conditions at a flow rate of 1 mL/min for 30 min. The alumina is then transferred from the extraction vessel to an empty SPE cartridge and the analytes are eluted with 6 mL methanol–water (65 + 35, v/v). Initial experiments used GC/MS of the HFBA derivatives to optimize the supercritical fluid extraction conditions, but further work was done to develop an LC/MS method for quantification and confirmation. The extract from the alumina was extracted with 2–6 mL volumes of *tert*-butylmethylether and, after drying the combined extracts at 40°C under a nitrogen stream, the residue was taken up in 0.050 mL methanol, which was then diluted with 0.150 mL water. A 0.10 mL aliquot was then injected on an LC/MS system equipped with an ion trap detector. Separations were on a reversed-phase column (2 micron particle size), with all target analytes eluting in 5–6 min. Using the ion trap, full mass spectra were obtained for each target compound, demonstrating that the predominant ion fragment obtained in the ACPI(+) mode was the protonated molecule. For screening purposes in the MS² mode, the [M+H–CH₃COOH]⁺ fragment was found to improve selectivity and these were subsequently used in the method development and validation (MA, 385; MPA, 387; MGA, 397; CMA, 405). The “parent” ions were used for screening purposes, while the additional ions required to meet confirmatory criteria were obtained monitoring transitions in MS² and MS³ modes. Using d₃-MGA as an internal standard, linear calibration curves were obtained over the concentration range 0.00025–0.0025 mg/kg for the 4 analytes. The limit of identification was 0.0005 mg/kg for each compound, with in-laboratory reproducibility <20% within the concentration range tested. The method was extended to screen for 3 additional compounds (screening ions in brackets), flurogestone acetate (*m/z* 347), delmadinone acetate (*m/z* 343), and fluprednisolone acetate (*m/z* 401). The method is currently used in the Dutch National Monitoring Program and fulfills EU criteria for residue control, while also providing quantitation and confirmation at the draft maximum residue limits for MGA currently under discussion within the Codex framework.

The analysis of nitrofurans and their bound metabolites continues to be of interest, particularly in the light of recent findings of these banned substances in survey samples by regulatory authorities in the European Union. Solid-phase extraction was used to recover residues of the protein-bound metabolite of furazolidone, 3-amino-2-oxazolidinone (AOZ), from pig liver (27). The method, tested on both fortified and incurred residues in tissues, removes the derivatizing agent used in the method as a potential source of interference in the determination, and also renders the method suitable for automation with either liquid chromatography or ELISA for extract analysis. Using the revised procedure, recoveries >60% of AOZ were obtained. A study was also undertaken to determine if the protein-bound metabolite of furazolidone, used to monitor for use of the banned substance in tissues, could also be ap-

plied to eggs (28). Residues of parent furazolidone and AOZ reached a plateau of 0.36–0.38 mg/kg on the fourth day of an 11 day administration of a ration containing 400 mg/kg furazolidone, with parent compound residues being primarily in the albumen. Residues of furazolidone were not detectable after 4 days withdrawal, but AOZ was detected up to 21 days and was distributed evenly between yolk and albumen. While furazolidone residues declined by 44% after storage at -20°C for 55 days, AOZ residues were stable. Residues of both furazolidone parent compound and AOZ were detected following extraction and cleanup by LC/MS using electrospray, with the m/z 243 fragment being used for quantitation of furazolidone and the m/z 253 fragment of the 2-nitrobenzaldehyde derivative of AOZ used to determine the metabolite. Recoveries were $>75\%$ for furazolidone and $>95\%$ for AOZ, with limits of detection of approximately 0.001 mg/kg for both compounds.

Gas chromatography with detection by negative ion chemical ionization mass spectrometry was used for the determination of another class of veterinary feed additives which have been banned from use in food animals in a number of countries, the 5-nitroimidazoles (29). The compounds tested were dimetridazole, ronidazole, ipronidazole, metronidazole and their hydroxy metabolites, applying the requirements for method validation as contained in the draft revised EU criteria for method validation. A 5 g test portion of muscle is homogenized for 4 min in a stomacher with 6 mL sodium chloride–potassium dihydrogen phosphate buffer and 1 mL of a protease solution, then hydrolyzed overnight at 37°C . The pH of the solution should be 3 for incubation. The digestate is centrifuged at 2600 g for 15 min at 4°C , the upper layer is decanted and the previous step was repeated after addition of 8 mL of the buffer. The upper layer was again decanted and the aqueous layers were combined and defatted with 2×10 mL hexane. After adjustment to pH 6, the aqueous layer volume was adjusted to 18 mL with buffer and loaded on an Extrelute[®] cartridge containing kieselguhr and, following a 20 min equilibration, the cartridge was washed with 20 mL ethyl acetate–*tert*-butylmethylether (1 + 1, v/v). Following a further 15 equilibration, the analytes were eluted with 2×20 mL ethyl acetate–*tert*-butylmethylether (1 + 1, v/v). The combined eluates were reduced in volume to 0.5 mL using a Turbovap, then evaporated to dryness under a nitrogen stream in a derivatization tube. Analytes were derivatized with a solution of 0.050 mL BSA and 0.050 mL *iso*-octane by heating at 50°C for 60 min to produce TMS derivatives. GC–MS analysis was conducted by injecting a 0.001 mL aliquot into an instrument equipped with a 30 m \times 0.25 mm id capillary column containing a 0.25 micron coating of methyl-5%-phenylsilicone at an injection block temperature of 285°C in splitless mode. The oven was programmed at a starting temperature of 85°C for 1.5 min, ramping at $15^{\circ}\text{C}/\text{min}$ to 100°C , then $5^{\circ}\text{C}/\text{min}$ to 140°C , $10^{\circ}\text{C}/\text{min}$ to 190°C , and finally $30^{\circ}\text{C}/\text{min}$ to 290°C , with a final hold time of 5 min. A carrier gas flow rate of 1 mL/min was used, with methane as the ionizing gas. Selected ion monitoring was used to detect the analytes, with 4 diagnostic ions being obtained for

dimetridazole, ronidazole, metronidazole and its hydroxy metabolite. Only 2 characteristic ions were obtained for ipronidazole and its hydroxy metabolite. Method validation was conducted using swine and turkey muscle (3 different sources for each) fortified with the analytes at 3 concentrations, with 7 replicates per concentration. While absolute recoveries varied from 15–25% for ipronidazole and its hydroxy metabolite to 45–60% for dimetridazole, 50–50% for metronidazole and its metabolite and 65–75% for ronidazole, the use of deuterated internal standards resulted in relative recoveries of about 100% for all the analytes. However, the low absolute recoveries for some analytes did have an effect on detection capability. The decision limits ($\text{CC}\alpha$) for dimetridazole, ronidazole, metronidazole and their hydroxy metabolites were 0.00065 to 0.0028 mg/kg, with relative standard deviations (in-laboratory reproducibility, 0.0005–0.004 mg/kg concentration range) from 6.4 to 20.3% at concentrations tested, but were 0.0052 mg/kg for ipronidazole and its hydroxy metabolite, with relative standard deviations of 9.2–34.4% at concentrations from 0.003–0.006 mg/kg. The methodology appears suitable for screening for concentrations in the 0.001 mg/kg range for these compounds and their metabolites and is capable of confirmation for most of the compounds at such concentrations.

Methodology was reported for the detection of a variety of antimicrobial drugs, including 6 sulfonamides, 3 nitrofurans and chloramphenicol, in pasteurized milk (30). The drugs were initially extracted with chloroform–acetone and, following evaporation of the extract to dryness, residues were dissolved in aqueous sodium acetate buffer and defatted by washing with hexane. The drug residues remained in the aqueous layer which was filtered and analyzed by liquid chromatography. The sulfonamides and chloramphenicol were separated on a reversed-phase column using a sodium acetate buffer–acetonitrile gradient, with UV detection of the analytes at 275 nm. Nitrofuran parent compounds were analyzed using isocratic conditions and detected at 375 nm. Recoveries from milk fortified at 0.050 mg/L ranged from 65% (sulfathiazole) to 94% (sulfamethazine) for the 6 sulfas tested (also included sulfamerazine, sulfachlorpyridazine, sulfamethazole, and sulfamonomethoxine), 104% for chloramphenicol, and were 72% for furaltadone, 100% for furazolidone, and 92% for nitrofurazone. Limits of detection ranged between 0.004 mg/L for nitrofurazone to 0.016 mg/L for sulfamethazine.

Results of monitoring of food samples of animal origin in Slovenia for chloramphenicol residues from 1991–2000 were reported, using capillary gas chromatography with electron capture detection (31). Quantitation was with reference to *meta*-chloramphenicol used as an internal standard. Recoveries were 89–102% for beef muscle and cow's milk, and 87% for whole eggs, at concentrations from 0.002–0.010 mg/kg, with a claimed limit of detection of 0.001 mg/kg. Out of 1308 random samples tested, there was only one positive finding, a milk sample which contained 0.0046 mg/kg. A procedure for the determination of chloramphenicol residues in milk using square-wave

voltammetry was also described (32). A linear response was obtained for concentrations corresponding to approximately 0.03–3.0 mg/L, with a detection limit of 0.015 mg/L and recoveries >97% from milk fortified at 0.064 and 0.32 mg/L.

The distribution of 5 sulfonamide drugs, sulfamethazine (sulfadimidine), sulfadiazine, sulfamethoxazole, sulfamonomethoxine, and sulfaquinoxaline was studied in laying hens administered the drug in feed at 100 mg/kg (33). Drug concentrations were monitored using liquid chromatographic analysis in plasma, muscle, and also in the liver, ovary and oviducts (the main tissues associated with egg production). Highest residues in all samples followed treatment with sulfaquinoxaline, while the lowest residues were after treatment with sulfamethazine. Residues of both these drug in liver were approximately double the concentrations found in muscle after 7 days of drug administration, while residues resulting from treatment with the other 3 drugs produced similar residue concentrations in both tissues. Significant residues were found in all cases in the tissues involved in egg formation, demonstrating a potential for residue transfer to the eggs if appropriate withdrawal times are not observed. The distribution of sulfamethazine and sulfadimethoxine residues in eggs following administration of these drugs to laying hens via drinking water was also reported (34). Both drugs were administered for 5 days, sulfamethazine at 0.5 g/L and sulfadimethoxine at 1 and 2 g/L. Albumen and yolk were analyzed by reversed-phase liquid chromatography after extraction and cleanup. Recoveries were 77–87% for the drugs in albumen and >65% in yolk, with a limit of quantitation of 0.005 mg/kg. There was an approximately 3:1 concentration factor in albumen relative to yolk, with residues requiring up to 20 days to fall below the limit of quantitation in albumen and 15 days in yolk. Highest residues were found on the fifth day of treatment, when residue concentrations for sulfadimethoxine were 35.7 mg/kg in albumen and 9.3 mg/kg in yolk, or 27.7 mg/kg on a whole egg basis, while sulfamethazine residues from the 2 g/L treatment were 70.4 mg/kg in albumen, and 44.5 mg/kg in yolk (62.1 mg/kg on a whole egg basis).

A method for the determination of trimethoprim, sulfadiazine, and the metabolite N^4 -acetylsulfadiazine in plasma of chickens includes deproteination of a 0.5 mL test portion with 1 mL acetonitrile, defatting with 2 mL hexane and extraction of the residue into 6 mL dichloromethane (35). After evaporation of the dichloromethane, the dried residue is dissolved in 0.5 mL mobile phase for determination by reversed-phase liquid chromatography with UV detection (240 nm for trimethoprim, 270 nm for sulfadiazine and N^4 -acetylsulfadiazine). Recoveries were >85% for the 3 analytes, with relative standard deviations <5%. Limits of quantification were in the range of 0.012–0.016 mg/L for the 3 analytes. The method is suitable for use in drug distribution and pharmacokinetic studies, but has not been validated for use on tissues.

A method for the determination of residues of sulfonamide drugs in eggs using LC–UV, with confirmation by LC/MS has also been reported (36). In this study, poultry were treated

with 15 drugs, including sulfamethazine, sulfadiazine, and sulfathiazole, to produce eggs containing incurred residues for method validation. Whole eggs were blended in an ice bath using a Polytron homogenizer, then a 5 g test portion was homogenized in an ice bath with 15 mL acetonitrile and centrifuged at 3000 g for 10 min at 0–4°C. The supernatant was removed and the process was repeated using 5 mL acetonitrile, the extracts were combined and 3 mL water was added. The volume was reduced to 1 mL under a nitrogen stream to remove acetonitrile, then the remaining extract was added to the reservoir of a C18 solid-phase extraction cartridge in which approximately 1 mL water remained after cartridge conditioning with acetonitrile and water. The cartridge was drained of liquid at a flow of 1–2 drops/s, then the sulfas were eluted with 3 mL acetonitrile. After addition of 1 mL water to the eluate, the volume was reduced to about 0.5 mL under a nitrogen stream, then the extract was diluted to 1 mL with water. Liquid chromatographic determinations were conducted using a C8 column and ternary gradient elution, using blends of 0.1% formic acid–methanol (90 + 10, v/v), methanol, and acetonitrile, with analyte detection at 287 nm. For LC–MS/MS confirmation using the ion trap detector, separations were on a C18 column using a multistep gradient of 0.1% aqueous formic acid and methanol. The MH^+ ions were isolated and full scan product ions were obtained for MS/MS confirmation. The determinative method was validated at concentrations from 0.050 to 0.200 mg/kg, while all 15 drugs could be confirmed at concentrations from 0.005–0.010 mg/kg.

Recoveries were >80% for all the sulfonamides except sulfacetamide (51–58%) at 0.050, 0.100, and 0.200 mg/kg concentrations. With the exception of sulfacetamide, relative standard deviations were $\leq 15\%$ for all compounds at the 3 concentrations tested. The determinative method was considered to meet FDA/CVM guidelines for all the sulfas tested except sulfacetamide, while the LC/MS/MS confirmation was considered acceptable for all drugs tested except sulfacetamide and sulfanilamide, which did not produce sufficient diagnostic ions.

Methodology using liquid chromatography with electrospray tandem mass spectrometry was reported for the determination of the benzimidazole anthelmintic drug mebendazole and its metabolites, hydroxymebendazole and aminobendazole, in sheep liver (37). A 1 g test portion of previously ground sample material was mixed on a Vortex mixer in a 50 mL centrifuge tube with 1 mL 0.1 mol/L sodium hydroxide, then shaken for 10 min with 20 mL ethyl acetate. After centrifugation at 5000 rpm for 5 min, the supernatant was decanted into a sidearm flask and the pellet was re-extracted with an additional 10 mL ethyl acetate. The ethyl acetate extracts were combined and evaporated to 4–5 mL, then transferred to a graduated tube, with a 2 mL ethyl acetate wash of the evaporating flask. The extract was then taken to dryness at 60°C under a nitrogen stream and the residue was dissolved in 0.600 mL methanol. This was washed with 2 mL hexane to defat the extract and the remaining methanol layer was adjusted to 1 mL by addition of methanol. LC–MS/MS analyses were conducted on a reversed-phase (C18) column using a

gradient elution containing varying proportions of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B). Detection used positive ion electrospray, with fragmentation of parent ions in the collision cell using argon. The parent ion and 2 daughter ions were monitored for each of mebendazole and its 2 metabolites. Flubendazole was used as an internal standard, as it is not licensed for use in sheep. Recoveries were >85% for the 3 analytes at the 3 concentrations tested (0.050, 0.100, and 0.400 mg/kg), with relative standard deviations from 5.4–11.9%. Decision limits and detection capabilities were calculated according to the draft EU criteria.

A method for the determination of residues of oxolinic acid and flumequine in freeze-dried salmon muscle with attached skin was reported as part of a project to develop a reference material (38). Freeze-dried tissue (0.5 g) was put into a 15 mL centrifuge tube, to which 2.0 mL 0.1 mol/L HCl was added, then 4 mL ethyl acetate. Test portions were homogenized for about 10 min using an agitator, then centrifuged for 5 min at 10 000 g. The supernate was transferred to a second 15 mL centrifuge tube and the extraction was repeated with 2 volumes of ethyl acetate (4 mL, followed by 2 mL), after which 4 mL 1 mol/L sodium hydroxide was added to the combined supernates, again with 10 min homogenization with the agitator, followed by centrifugation at 10 000 g for 5 min. The supernate was transferred to another 15 mL centrifuge tube, and the extraction was repeated with 4 mL 1 mol/L sodium hydroxide, followed by 1 mL 85% *ortho*-phosphoric acid solution, then 3.5 mL chloroform. The homogenization and centrifugation steps were repeated and the chloroform extract was transferred to a 10 mL glass tube. The extraction was repeated with a second 3.5 mL chloroform, the combined extracts were taken to dryness under a nitrogen stream at 40°C and the residue was taken up in mobile phase. Analysis was by liquid chromatography under isocratic conditions on a reversed-phase column using a mobile phase consisting of 0.02 mol/L aqueous *ortho*-phosphoric acid–acetonitrile (67 + 33, v/v), with detection by fluorescence (excitation 325 nm, emission 365 nm). Limits of detection and quantitation for the freeze-dried tissue were 10 ng/g for oxolinic acid and 50 ng/g for flumequine (3 and 16 ng/g, respectively, on a wet weight basis).

Solid-phase extraction followed by capillary electrophoresis with UV detection was applied to the determination of the fluoroquinolone antibiotics difloxacin and sarafloxacin in chicken muscle (39). A 5 g test portion was homogenized with 5 mL 0.05 mol/L phosphate buffer adjusted to pH 7.0 with 3N NaOH, then extracted with 2 volumes (20 mL, then 10 mL) dichloromethane. After 20 min shaking with a rotary shaker and centrifugation at 1467 g for 5 min, the organic extract was transferred to a 40 mL centrifuge tube and washed with 2–5 mL volumes of 0.5N NaOH, then centrifuged again at 1467 g for 5 min to separate the organic and aqueous layers. The aqueous phase was transferred to a clean tube, neutralized by addition of 15 mL 0.002 mol/L phosphoric acid, then defatted by addition of 10 mL hexane. The solution was again centrifuged at 1467 g for 5 min, after which the aqueous phase was loaded onto a C18 solid-phase extraction cartridge which

had previously been activated by washing with 2 mL methanol, followed by 2 mL water and finally 2 mL 0.050M phosphate buffer (pH 7). After sample loading, cartridges were rinsed with 2 mL water, followed by 0.5 mL acetonitrile, then the fluoroquinolones were eluted with 2 mL 4% trifluoroacetic acid in water–acetonitrile (25 + 75, v/v), followed by 1 mL acetonitrile. The eluate was evaporated to dryness under a stream of nitrogen at 50°C and the residue was taken up in 0.1 mL acetonitrile–water (1 + 1, v/v) for injection into the capillary electrophoresis system. The capillary electrophoresis system used as mobile phase a buffer solution of 0.025M diethylmalonic acid adjusted to pH 8.22 with 3M NaOH and a capillary column (50 cm in length, 0.075 mm id) maintained at a temperature of 25°C. The separation potential was 20 kilovolts and the analytes were detected at 275 nm. A linear response was obtained for both compounds from 0.050–0.300 mg/kg, with detection limits of 0.010 mg/kg for difloxacin and 0.025 mg/kg for sarafloxacin. The related compound marbofloxacin is used as an internal standard.

Liquid chromatography with electrospray mass spectrometric detection was applied to the determination of the fluoroquinolone norfloxacin in poultry muscle (40). A 1 g test portion of muscle is homogenized with 2 mL methanol–acetic acid (98 + 2, v/v). After centrifugation at 1300 g for 10 min, the supernate is transferred to a second tube and the liquid is evaporated. The dried residue is taken up in 1 mL methanol–acetic acid (98 + 2, v/v), to which is added 2 mL acetonitrile–saturated hexane. After mixing on a Vortex mixer and centrifugation at 1300 g for 10 min, the upper layer is discarded and the lower layer is evaporated to dryness. The dried residue is taken up in 1 mL methanol–acetic acid (98 + 2, v/v) and analyzed by reversed-phase liquid chromatography using a gradient mixture of 2 components, water–acetic acid (98 + 2, v/v) and acetonitrile, at a flow rate of 0.4 mL/min. Norfloxacin is detected by monitoring for the $[M+H]^+$ ion at m/z 320. Recoveries were >80% in tissues fortified at 0.010 and 1.0 mg/kg, with relative standard deviations 9–12%. The estimated limit of quantitation was 0.005 mg/kg.

An alternative approach to the rapid detection of the fluoroquinolone enrofloxacin was the use of an ELISA assay, including the development of an immunochromatography-based rapid test (41). The ELISA had a detection limit of 0.001 mg/kg for enrofloxacin residues in milk and 0.010 mg/kg for residues in chicken liver and muscle. Recoveries from the tissues were 72–96%, while recoveries from milk were 84–99%. Detection limits using the immunochromatographic test kit were 10-fold higher. Analytical results obtained with the ELISA had a high correlation (>0.98) with analytical results obtained using a liquid chromatographic assay for residues in chicken liver and muscle, and for milk.

The use of tetracyclines in aquaculture continues to be of interest. Methodology was described for the determination of oxytetracycline (OTC) residues in various matrixes, including trout muscle, biofilter sand, sediment and tank water from a recirculating aquaculture system (42). Various approaches to extraction and cleanup were used, depending on the sample matrix and the detection limit required. Water (0.5–1.0 mL)

was diluted with an equal volume of McIlvaine–EDTA buffer and filtered into an autosampler vial for liquid chromatographic analysis. Sand (1 g) was shaken vigorously with 25 mL 0.1N hydrochloric acid for 30–45 min, then centrifuged at 3500 g for 10 min. An aliquot of the supernate was filtered into an autosampler vial for analysis. Sediment (2 g) was extracted by homogenizing with 25 mL McIlvaine–EDTA buffer, then shaking 2 h on a platform shaker. The mixture was then centrifuged at 3500 g for 10 min and an aliquot of the supernate was filtered into an autosampler vial for samples expected to contain <100 mg/kg of OTC, while aliquots of samples expected to contain >100 mg/kg OTC were diluted 100-fold prior to analysis. For trout muscle, extraction was based on that used in the AOAC Official Method for mammalian tissue (Method 995.09), which includes extraction with McIlvaine–EDTA buffer, followed by cleanup on C18 solid-phase extraction cartridges (43). Liquid chromatographic analyses for extracts from the various sample matrixes were conducted using a polymeric column (PLRP-S, 5 micron) held at 40°C, with a 0.1% aqueous trifluoroacetic acid–acetonitrile gradient. Limits of detection were 0.04 mg/kg for trout muscle, 0.03 mg/kg for sand, 1 mg/kg for sediment and 0.003 mg/L for water. This study is illustrative of the growing concerns shifting the focus of analytical methods from drug residues as potential contaminants in animal-derived foods to potential environmental contaminants, with a resulting need for method validations for “nontraditional” matrixes.

Methodology for the determination of OTC and its 4'-epimer (4-*epi*-OTC) in turkey muscle, liver, and kidney using an isocratic reversed-phase liquid chromatographic separation was also reported (44). A 1 g test portion of prehomogenized tissue was stirred with 7.5 mL McIlvaine buffer (pH 4) for 10 min in a centrifuge tube, then 7.5 mL methanol was added and, after mixing on a Vortex mixer, the contents were centrifuged at 10 000 g for 10 min at 4°C. The supernatant was transferred to a second tube and a 6 mL aliquot was loaded onto a previously prepared extraction cartridge. A cleanup procedure similar to that used for milk in Official Method 995.05 was used (43). Cartridges were prepared by slowly adding chelating Sepharose Fast Flow Gel to an empty reservoir containing a frit to a bed height of 1 cm, then placing a second frit on top of the bed. The bed was washed with 3 × 0.5 mL water, followed by 0.5 mL 0.010M cupric sulfate solution, then a further 0.5 mL water. At this point, there should be a uniform blue layer in the upper third of the resin bed. Load the 6 mL aliquot of sample extract (corresponding to 0.4 g tissue), then wash the cartridge sequentially with 0.5 mL water, 0.5 mL methanol, 0.5 mL water, then 0.1 mL McIlvaine–EDTA elution buffer (pH 4, prepared by mixing 614.5 mL 0.1 mol/L aqueous citric acid solution with 380 mL 0.2 mol/L aqueous Na₂HPO₄ solution and 37.2 g EDTA disodium salt). Elute analytes with 0.8 mL McIlvaine–EDTA buffer into graduated 10 mL glass tube. Dilute liver and kidney extracts to 1 mL with buffer. Inject 0.100 mL aliquot of extract into liquid chromatograph with C18 reversed-phase column (15 cm × 4.6 mm id, 5 micron packing) with mobile phase consisting of acetonitrile–0.01 mol/L aqueous oxalic

acid solution (85 + 15, v/v) at 1 mL/min flow rate, with UV detection at 355 nm. No interfering peaks were observed from other tetracyclines. Recoveries were 70–96% for 4-*epi*-OTC and 71–89% for OTC from all 3 tissues at the concentrations tested (0.0125–0.300 mg/kg for 4-*epi*-OTC; 0.050–1.200 mg/kg for OTC). Validation was conducted at concentrations bracketing maximum residue limits established as sum of the parent compound and epimer by the European Union (0.100 mg/kg for muscle; 0.300 mg/kg for liver; 0.600 mg/kg for kidney). Tissues from turkeys which received OTC via drinking water for 3 days at 400 mg/L collected and analyzed after 1, 3, and 5 days withdrawal demonstrated that residues were below the MRLs after 3 days withdrawal. The 4-*epi*-OTC residues were <15% of the total residues in all samples and were not detectable in muscle samples at 3 and 5 days withdrawal, or in liver samples at 5 days withdrawal.

The determination of residues of the macrolide antibiotics erythromycin and oleandomycin in meat, fish, milk, and eggs using liquid chromatography with fluorescence detection after formation of 9-fluoromethylchloroformate (FMO) derivatives has been reported (45). Meat, fish, and eggs (10 g) are homogenized in a centrifuge with 20 mL acetonitrile, centrifuged at 1480 g and the acetonitrile liquid phase is removed and evaporated. The residue is taken up in 20 mL 0.1 mol/L citrate buffer (pH 6.0) and defatted with 20 mL hexane. After centrifugation to break up any emulsion formation, a 10 mL aliquot is collected for further cleanup. Milk (100 mL) is defatted by centrifugation at 5300 g at 5°C for 30 min, then a 25 mL aliquot of the defatted milk is mixed with 25 mL 0.1 mol/L citrate buffer and 25 mL hexane. After 15 min shaking on a mechanical shaker, the mixture is centrifuged at 1480 g for 5 min and a 25 mL aliquot of the aqueous phase is removed for further cleanup. A common cleanup is used for all extracts, which are loaded onto cation exchange cartridges previously conditioned with 5 mL 0.1 mol/L citrate buffer. Samples are loaded at a flow rate of approximately 1 drop/s, then the cartridges are washed with 2 mL water, followed by 1 mL methanol. After the cartridge has dried for 5 min, 3 mL 0.1 mol/L phosphate buffer (pH 9.3) are added, then the macrolides are eluted with 3 mL methanol. The methanol eluate is collected, dried and the residue taken up in 2 mL of the 0.1 mol/L phosphate buffer. The macrolides are then extracted into dichloromethane (2 × 2 mL) and dried under nitrogen. The residue is taken up in 0.1 mL acetonitrile, to which 0.1 mL 0.4 mg/mL FMO solution (in acetonitrile) and 0.1 mL 0.1 mol/L phosphate buffer (pH 7.5) were added. After heating at 45°C for 60 min, 0.2 mL 0.03 mol/L phosphate buffer (pH 7.0)–acetonitrile (1 + 1, v/v) was added and derivatized extracts were kept in amber vials until analyzed. Gradient analysis was performed on a reversed-phase liquid chromatography column using a mixture of acetonitrile and 0.03 mol/L phosphate buffer (pH 7.0)–acetonitrile (36 + 64, v/v) at a flow rate of 1 mL/min. Derivatives are detected using an excitation wavelength of 260 nm and emission wavelength of 305 nm. To correct for recoveries which are in the 50% range, roxithromycin was used as an internal standard. Limits of quantitation were 0.1 mg/kg for erythromycin

in meat (muscle, liver, kidney), fish, and egg and 0.05 mg/kg in milk, while the comparable results for oleandomycin residues were 0.1 mg/kg for meat (muscle), fish, egg, and milk, and 0.20 mg/kg in liver and kidney. Repeatability varied from 3.6 to 12.9% for the 2 compounds across the range of matrixes tested and a linear response was observed for concentrations of 0.1 to 1.0 mg/kg of the residues and a single chromatographic peak was observed for each target drug following derivatization.

Residues of the macrolide antibiotic tylosin were determined in muscle, liver, kidney, and fat (with adhering skin) of calves, pigs, and poultry using liquid chromatography with UV detection at 280 nm (46). Test portions of muscle (5 g) were homogenized with 10 mL chloroform and 1 mL 0.07 mol/mL phosphate buffer (pH 8.5), shaken for 20 min, then centrifuged at 1932 g for 20 min. The chloroform layer was removed, dried and the residue dissolved in 4 mL acetonitrile–water (8 + 2, v/v). Following partitioning with 10 mL dichloromethane, the mixture was shaken, centrifuged and the dichloromethane layer removed. The repartitioning was repeated with 5 mL dichloromethane and the combined extract was dried, then taken up in 0.2 mL mobile phase for analysis. A similar procedure was used for liver and kidney, substituting 7N NaOH and 50% NaOH, respectively, for the phosphate buffer during the initial extraction. Poultry fat (5 g) was homogenized with 0.1 mL 7N NaOH and 10 mL ethyl acetate, centrifuged at 1932 g for 20 min, then the organic phase was removed and evaporated. The oily residue was dissolved in 4 mL methanol, centrifuged and the methanol was removed and evaporated to dryness. The residue was dissolved in 0.2 mL mobile phase. For calf fat, the volume of ethyl acetate used was increased to 15 mL. For pig fat, 2 mL 0.07 mol/mL phosphate buffer (pH 8.5) was substituted for the 7N NaOH. Albendazole was used as an internal standard for the analysis of muscle, liver, and kidney, but not for fat. Extracts were analyzed by reversed-phase liquid chromatography using a mobile phase consisting of acetonitrile–aqueous 0.04M Na₂PO₄ (1 + 2, v/v) at a flow rate of 1.5 mL/min. A limit of quantitation of 0.05 mg/kg was achieved, with a linear range of 0.050–0.50 mg/kg for muscle and kidney, 0.05–0.30 mg/kg for liver and pig fat, and 0.05–0.40 mg/kg for poultry fat. The method was applied to incurred samples obtained from 8 calves administered with tylosin at 20/mg/kg body weight/day for 5 days, then slaughtered at 7 and 14 days of withdrawal. Tylosin residues were stable in spiked kidney tissues stored at –80°C for 400 days, and in other tissues stored 80–120 days prior to re-analysis.

Residues of the macrolide antibiotics tylosin and erythromycin were also determined in bovine muscle using an indirect assay based on ELISA with electrochemical detection (47). Monoclonal antibodies were used as BSA conjugates to compete with free analyte, after which the activity of the horseradish peroxidase-labelled antiglobulins was determined electrochemically. Limits of detection were 0.0004 mg/L for erythromycin and 0.004 mg/L for tylosin. Other macrolides did not interfere in the assay, except for

roxithromycin, which is derived from erythromycin. Results of this screening test were confirmed by LC/MS/MS.

An LC/MS/MS method was applied to identify and confirm residues of β -lactam antibiotics detected in bulk raw milk (tanker) using several test kits, based on receptor and microbial growth inhibition detection principles (48). Of 18 suspect samples tested, 16 contained detectable residues using the LC/MS/MS assay, with residues of 2 drugs found in 4 samples and 3 drugs detected in one sample. Penicillin G was detected in 8 samples, with 2 of these exceeding the MRL of 0.004 mg/kg; cloxacillin residues were found in 7 samples, with one exceeding the MRL of 0.030 mg/kg; amoxicillin residues were found in 4 samples, with one above the MRL of 0.004 mg/kg; ampicillin residues were detected in 3 samples, none above the MRL of 0.004 mg/kg. In the LC/MS/MS method, d₇-penicillin (potassium benzyl-(d₇-phenyl) penicillate) is added as an internal standard to a 20 mL test portion of milk which is mixed on a Vortex mixer, then centrifuged at 13 800 g for 30 min at 4°C (49). A 12 mL aliquot of the supernate is mixed on a Vortex mixer for 1 min with 12 mL 0.1 mol/L phosphate buffer (pH 9.2), then 12 mL hexane is added and shaken 10 min. The mixture is then centrifuged at 2790 g for 10 min at 10°C and a 15 mL aliquot of the aqueous phase is loaded onto a C18 solid-phase extraction cartridge which has been previously conditioned with 10 mL methanol, 10 mL water, and 5 mL 2% aqueous sodium chloride solution. Wash the cartridge after loading with 2 mL 2% aqueous sodium chloride solution, followed by 2 mL water, then elute the analytes with 1.5 mL acetonitrile–0.050 mol/L phosphate buffer, pH 8 (1 + 1, v/v). The eluate is evaporated to <0.75 mL, then 0.070 mL 1% formic acid is added and the volume is adjusted to 1 mL by addition of water. After filtration through a 10 000 Da cut-off device at 7000 g, a 0.010–0.015 mL aliquot is analyzed by LC/MS/MS using electrospray ionization, positive ion mode. Identification is based on a parent ion plus 2 or 3 transition ions for each target analyte. Confirmation limits are as follows: penicillin G, 0.00053 mg/kg; ampicillin, 0.00052 mg/kg; amoxicillin, 0.00042 mg/kg; cloxacillin, 0.0011 mg/kg; oxacillin, 0.00040 mg/kg.

Residues of the β -blocker drug carazolol in porcine muscle were determined using a radioreceptor assay in which carazolol residues competed for receptor sites with [³H]-dihydroalprenolol (50). Residues are extracted under basic conditions into ether for analysis. Limits of detection were approximately 0.001 mg/kg in pig muscle and 0.0015 mg/kg in pig kidney. In a study to determine if carazolol residues persist for up to 30 h post-injection at the recommended dose of 0.010 mg/kg body weight, highest residues were found at 1 h (0.011 mg/kg in kidney, 0.004 mg/kg in muscle), but these were below the MRLs established for this compound by the European Union (0.005 mg/kg in muscle, 0.025 mg/kg in kidney). The method was considered to be suitable for use as a screening method.

A time-resolved fluorescence immunoassay (TR-FIA) was reported for the determination of zeranin in bovine urine, using immunoaffinity chromatography for sample cleanup and

specificity-enhanced zeranol antibody to reduce cross-reactivity with related *Fusarium* spp. toxins (51). The dry-well chemistry approach with the reagents present in the reaction well in dry form resulted in a simple assay, requiring only the addition of the sample extract and a 1 h reaction time before results were read. A limit of detection of 0.0013 mg/L was calculated from analysis of 20 blank urine samples. At 0.002 mg/L zeranol, recoveries of 99% were obtained, with a relative standard deviation (within assay) of 4.5–9%.

Interest in the determination of other hormonal substances in bovine urine continued, with publication of a method for some of the more difficult analytes, including 17 α -trenbolone, β -boldenone, 16- β -OH-stanozolol, and 4-chloroandrost-4-ene-3,17-dione, based on LC/MS/MS (52). A 20 mL test portion of urine is pH adjusted to 4.6 by addition of 3 mol/L acetate buffer (pH 4.6), then loaded on a C18 solid-phase extraction cartridge. The cartridge was washed with 2 \times 5 mL water, then the analytes were eluted with 2 mL methanol. The eluate was dried under nitrogen and the residue was dissolved in 0.100 mL methanol, to which 5 mL 0.2 mol/L acetate buffer (pH 4.6) and 0.050 mL *Helix pomatia* digestive juice were added. After incubation at 60°C for 2 h, the extracts were centrifuged at 1100 g for 10 min, the supernatant was loaded on a C18 solid-phase extraction cartridge and washed with 2 \times 5 mL water. An NH₂ cartridge was then attached to the outlet of the C18 cartridge and the analytes were eluted with 2 mL ethyl acetate. The eluate was evaporated to dryness under a nitrogen stream, then dissolved in 0.150 mL mobile phase (methanol–water–formic acid, 64.7 + 34.7 + 0.6, v/v/v). A precursor ion and 3 product ions were selected for each target analyte and validation was conducted according to the draft EU criteria. Specificity was established by analysis of 20 blank urine samples, then analysis of the blank urine fortified with a number of other hormonal substances implicated in unauthorized usage. No false negative results were obtained from 20 blank samples spiked at 0.001 mg/L.

An improved method using LC/MS/MS with electrospray was reported for residues of the carbadox marker residue, quinoxaline-2-carboxylic acid, in pig liver (53). The use of carbadox as a feed additive has been prohibited in a number of countries, resulting in a requirement to develop methods with lower detection limits than those used to monitor compliance with a maximum residue limit of 0.030 mg/kg which was previously in effect. A 5 g test portion of homogenized liver was weighed into a 35 mL tube and internal standard was added to each sample (d₄-QCA). After 10 min equilibration, 10 mL 3 mol/L sodium hydroxide was added to the tube, which was transferred to a water bath to incubate for 30 min at 100°C. After cooling to room temperature, 4 mL concentrated hydrochloric acid was added and the contents were mixed for 30 s, then 6 mL ethyl acetate were added. The tubes were inverted for 1 min, then centrifuged at 2000 g for 10 min at 4°C. The aqueous layer was transferred to a 10 mL glass tube containing 1 mL concentrated hydrochloric acid and loaded onto a solid-phase extraction cartridge containing a nonendcapped benzenesulfonic acid as the chromatographic medium. After washing with 5 mL 0.1 mol/L hydrochloric acid, the analyte

was eluted with 3 mL 0.1 mol/L sodium hydroxide–methanol (70 + 30, v/v) into a 10 mL glass tube. After addition of 0.300 mL concentrated hydrochloric acid and 2 mL ethyl acetate and mixed on a Vortex mixer for 15 s, the tubes were centrifuged at 2000 g for 10 min at 4°C and the upper (ethyl acetate) layer was then transferred into a glass centrifuge tube. The extraction was repeated twice and the ethyl acetate extracts were combined and taken to dryness under nitrogen at 60°C. The residue was dissolved in methanol–water (10 + 90, v/v) and mixed on a Vortex mixer for 15 s, then transferred to microvials for analysis using a reversed-phase chromatographic column with a mobile phase of methanol–water–glacial acetic acid (40 + 59.6 + 0.4, v/v/v) at a flow rate of 1 mL/min, split so that about 0.20 mL/min was directed to the mass spectrometer. An injection volume of 0.030 mL was used. The first stage of the mass spectrometer was set to transmit the pseudo-molecular ions of QCA and the internal standard ($[M+H]^+$ m/z 175, 179), while the second quadrupole transmitted the product ions m/z 129, 102, and 75 for QCA and 106 for d₄-QCA. Concentrations of QCA were calculated by comparing the ratio of the 175/129 response with that of the 179/106 ions for d₄-QCA. The method was validated according to the draft EU criteria over the concentration range 0.003–0.300 mg/kg. The decision limit CC α was calculated to be 0.00016 mg/kg, while the detection capability CC β was 0.00027 mg/kg.

A major conference on veterinary drug issues with a focus on analytical methods, the 4th International Symposium on Hormone and Veterinary Drug Residue Analysis, was held in Antwerp from June 4–7, 2002. Over 30% of the over 200 oral presentations and posters reported on LC/MS and LC/MS/MS applications, indicating the dominant role these techniques are assuming in drug residue laboratories today. A selection of papers presented at this symposium are scheduled for publication in a special issue of *Analytica Chimica Acta*.

Discussion of requirements for validation of analytical methods to support maximum residue limits under consideration by the Codex Committee on Residues of Veterinary Drugs in Foods continued at the 13th Session of the Committee, held in Charleston, NC, December 3–7, 2001. Developments in approaches to single laboratory validation and the performance-criteria approach to method recognition were discussed and continue to be monitored, with a drafting party preparing further material on the subject of method validation for consideration at the 14th Session of the Committee. Regulatory analysts engaged in import–export testing of foods should be aware of this discussion and can find relevant information on harmonized guidelines for single laboratory validation of analytical methods recently published by the International Union of Pure and Applied Chemistry (54). Guidance on the application of these guidelines to residue methods for veterinary drugs is contained in a consultation report available at the Web site of the International Atomic Energy Agency Training Centre (55) or published in a recent symposium proceeding (56).

Recommendations

(1) *Residues of Triclabendazole in Liver, LC Method*: Study Director Herbert Koch, Swiss Federal Veterinary Office, Schwarzenburgstrasse 161, Liebefeld 3097-CH, Switzerland, Tel: +41-31-323-8522, Fax: +41-31-323-8522, E-mail: Herbert.Koch@bvet.admin.ch. Discontinue topic.

(2) *Improved Analysis Of Tetracycline Residues in Swine Tissues Using Polymer-Based Extraction Cartridges*: Study Director Jan Smudzki, National Veterinary Research Institute, Al Partyzantow 57, Pulawy 24100, Poland, Tel: +48-81-866-3051, Fax: +48-81-866-2595, E-mail: zmudzki@piwet.pulawy.pl. Continue study. The General Referee considers that the method as published, involving a study by 5 laboratories, will fit in the proposed e-CAM[®] database in the category of "multilaboratory validated methods." No further work is planned. Discontinue topic.

(3) *Determination of Clopidol in Chicken Tissues*: Study Director Guo-Fang Pang, Qinhuangdao Entry-Exit Inspection and Quarantine Bureau of P.R. China, No. 39 Haibin Rd, Qinhuangdao 06600-2, People's Republic of China, Tel/Fax: +86-335-340-7608, E-mail: panggfciq@pang.com.cn. The report is under review by the Committee and the method will be considered for Official First Action upon completion of the review. Continue study.

(4) *β -Lactam Antibiotics in Milk, LC Method*: Vacant. Continue study. Seeking new Study Director. Any scientist or organization interested in participating in this topic is asked to contact the General Referee or AOAC INTERNATIONAL.

(5) *Determination of Ivermectin in Animal Tissues*: Vacant. Continue study. Seeking new Study Director. Any scientist or organization interested in participating in this topic is asked to contact the General Referee or AOAC INTERNATIONAL.

(6) *Application of Optical Immunobiosensor Technology to the Determination of Veterinary Drug Residues in Foods*: Establishment of a new topic and appointment of a Topic Advisor is recommended. Any scientist or organization interested in participating in this topic is asked to contact the General Referee or AOAC INTERNATIONAL.

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