



Residue depletion of ivermectin in broiler poultry

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ABSTRACT

Helminth infections are widespread in the poultry industry. There is evidence of extra-label use of some drugs, such as ivermectin (IVM), in broiler poultry. Pharmacokinetic and residual studies of IVM in poultry, however, are rather scarce. Our aim was to determine time restrictions for broiler chickens fed with balanced feed mixed with IVM for 21 days, and thus achieve acceptable residual levels for consumption as established by the European Union. Sixty 1-day-old chicks were fed with food supplemented with IVM at 5 mg kg⁻¹ feed for 21 days. Groups of six treated animals were sacrificed at 0, 1, 2, 4, 8, 10, 15, 20 and 28 days after treatment. Liver, skin/fat, kidney and muscle samples were obtained. IVM were determined by liquid chromatography with fluorescence detection after automatic solid-phase extraction with SPE C₁₈ cartridges. The highest concentrations were measured in the liver, which is logical given that IVM is a drug that undergoes extensive hepatic metabolism. The optimal withdrawal time for edible tissues of these animals to stay within the permitted residual levels were: 12 days for liver, 8 days for skin/fat, 0 days for muscle and 10 days for kidney.

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Introduction

Domestic birds are frequently affected by internal parasites (*Capillaria* spp., *Ascaridia* spp., *Heterakis gallinarum*, *Syngamus trachea*) and external parasites (*Dermanyssus gallinae*, scabies mites – *Cnemidocoptes mutans*, fleas – *Ceratophyllus gallinae*, and some ticks – *Argas persicus*) (Sharma et al. 1990; Bennett & Cheng 2012). Another ectoparasite of great economic impact on the poultry farm is the beetle poultry litter, *Alphitobius diaperinus*, with a significant negative impact on performance and production of birds. *Ascaridia galli* is an important nematode parasite of poultry; heavy infection causes death of the chickens, whereas moderate to low infection interferes with growth and productivity (Sharma et al. 1990). The control of this nematode infection using anthelmintics through medicated feed or water has reduced losses in the poultry industry. Currently, formulations based on ivermectin (IVM) for the treatment of parasitic infections of birds are available on the market, but they have not yet been approved for use in avian species and, therefore, there are still no studies on

the profile of tissue depletion of these molecules in chickens destined for human consumption.

It is well established that the efficacy of any anthelmintic drug depends not only on its affinity for specific parasite target sites but also on its ability to reach high and sustained drug concentrations where the parasites are located. IVM is one of the most useful anti-parasitic agents (Mestorino et al. 2003). It is a member of the macrocyclic lactone family known as avermectins (AVM) derived from *Streptomyces avermitilis* (Echeverría et al. 2002), endectocides of wide spectrum of activity at low doses rates with high potency and low mammalian toxicity (Galarini et al. 2013) widely used for treatment and prevention of internal and external parasites in food-producing animals (Mestorino et al. 2003). IVM is effective when administered orally, parenterally or topically, and absorption is rapid by any of these routes (Echeverría et al. 2002). This compound – when administered in water – was effective in removing *Ascaris galli*, *Heterakis gallinarum* and *Capillaria* spp. in poultry (Schepkins et al. 1985; Sharma et al. 1990; Todisco et al. 2008; Khayatnouri et al. 2011). It is a very lipophilic molecule, so residues remain for long

periods in the treated animal tissues, especially those with a high fat content (Baynes et al. 2000). Until recently, there were some oral formulations to control internal and external parasites in game birds and fighting cocks in some countries, but most are no longer available on the market. In addition, no IVM formulations for avian production were available. However, extra-label use of this drug has been reported (Bennett & Cheng 2012).

Residue studies are of fundamental importance in public health. Consumer safety is based on a series of measures including MRLs and ADIs as the most important. MRLs have been established by Commission regulations 37/2010, 418/2014 and 1390/2014 (EU 2010, 2014a, 2014b) for some AVMs in specific animal tissues (bovine, ovine, caprine, equine, fish), but there are no established MRLs for edible tissues of chicken. The European Union set the MRLs for IVM in edible tissues of food-producing mammals as follows: 100 ng g⁻¹ in liver and fat, and 30 ng g⁻¹ in kidney and muscle. Extrapolation of MRLs to the relevant minor species has been considered.

The aim of this study was to determine restriction periods for broiler chickens fed with a balanced feed mixed with premix containing IVM for 21 days in order to establish acceptable residual levels for human consumption according to European Union regulations.

Materials and methods

Reagents and chemicals

Ivermectin (IVM) pure reference standard (97% purity) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Acetonitrile and methanol solvent used during the extraction and drug assay were of HPLC grade and purchased from J. T. Baker (Phillipsburg, NJ, USA). The N-methylimidazole and trifluoroacetic anhydride used for the derivatisation reaction were from Sigma-Aldrich Chemical Co. (Milwaukee, WI, USA). Solid-phase extraction (SPE) columns (Strata, C₁₈, 100 mg, 1 ml) and analytical column (Kinetex C₁₈) were obtained from Phenomenex (Torrance, CA, USA).

Study design treatment and administration

It is a common practice in the poultry industry to dose chickens by adding IVM to the feed or in the

water system. Sixty-one-day-old BB chicks were fed with a pre-start and initiator feed supplemented with IVM at 5 mg kg⁻¹ for 21 days. This means that if in 21 days a chicken consumes 1 kg of feed during the same time it also consumes 5 mg of IVM (238 µg day⁻¹). We were not able to measure feeding rates in this study. However, according to Cobb-Vantress (2010), chicks of 1, 7, 14 and 21 days old eat approximately 13, 28, 68 and 111 g day⁻¹. Assuming this intake, chicks in this study would have approximately consumed 65, 140, 340 and 555 µg of IVM, respectively. Considering the chicks' body weight: 185 g (1–7 days), 465 g (7–14 days) and 943 g (14–21 days), the estimate daily IVM dosage was between 757 and 588 µg kg⁻¹.

The chickens treated with IVM were euthanised by cervical dislocation after desensitisation by passage of an electric current through the head in groups of six animals at 0, 1, 2, 4, 8, 10, 15, 20 and 28 days post-treatment. The last time corresponds to the sacrifice for commercial purposes. Six chickens used as control (free of IVM) were euthanised before the experiment. Immediately after slaughter, liver, skin/fat, muscle and kidney samples were collected. Each sample was properly conditioned, placed in plastic bags, heat sealed, labelled and stored at -20° C until assay.

The protocol followed the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (Federation of Animal Science Societies – FASS) and was approved by the Experimental Ethics Committee of the Faculty of Veterinary Science, UNLP, Argentina.

Ivermectin analysis

All tissue samples were analysed by HPLC with automated SPE and fluorescence detection following an adapted version of the methodology previously described (Echeverría et al. 2002; Mestorino et al. 2003). Tissue samples (muscle, liver, kidney and skin plus fat) were thinly sliced and 2 g were homogenised in 1 ml of acetonitrile (Ultra Turrax T₂₅ basic, IKA, Works Inc., Lincoln, NE, USA). The homogenate was mixed for 20 min, sonicated for 10 min (ultrasound bath) and centrifuged at 2000g for 10 min at 4°C. The clear supernatant was transferred to a new tube and the extraction procedure was repeated once. The total supernatant obtained was

placed on the appropriate rack of Aspec XL automatic SPE apparatus (Gilson, Villiers-le-Bel, France). Automatic sample preparation was performed using SPE C₁₈ cartridges (Strata C₁₈, 100 mg, 1 ml, Phenomenex), which were conditioned with 2 ml of methanol, and followed by 2 ml of water HPLC quality. All samples were applied to cartridges, washed with 1 ml of water followed by 1 ml of methanol/water (1:3, v/v), dried with air for 2 min and finally eluted with 3 ml of methanol of HPLC grade. The eluted volume was evaporated at 60°C to dryness in a vacuum concentrator (AVC 2-25CD Christ, Osterode, Germany). A fluorescent derivative was obtained by dissolving the dry residue in 100 µl of a mixture of N-methylimidazole solution in acetonitrile (1:1, v/v). To initiate the derivatisation, 150 µl of a solution of trifluoroacetic anhydride in acetonitrile (1:2, v/v) were added. After completion of the reaction (< 30 s) and mixing, the solution was transferred to autosampler polypropylene vials and an aliquot of 100 µl was injected into the chromatographic system. HPLC analyses were carried out within 4 h to avoid the degradation of the fluorescent derivatives.

Standard curve

Standards were prepared by adding 0.5, 1, 2.5, 5, 10, 20 and 30 ng of IVM to test tubes, evaporating to dryness at 60°C, and dissolving and derivatising as described above. Linear regression analysis using a least-square fit was performed.

Chromatographic conditions

The chromatographic system consisted of an isocratic pump (Gilson Inc. 307), an automatic injector (Gilson Inc. 234), a FluoroMonitor IM III Detector (Sp Thermo Separation products) set at an excitation wavelength of 365 nm and an emission wavelength of 475 nm, and Eppendorf CH-30 Column Heater (set at a 30°C). The system is controlled through the Unipoint® Gilson system. An C₁₈ column (Kinetex, 2.6 µm, 4.6 mm × 100 mm; Phenomenex) and was eluted with a mixture of acetic acid 2% in water-methanol-acetonitrile (4:32:64) at a flow rate of 1.5 ml min⁻¹ at 30°C. Identification of IVM in bird tissues was accomplished by comparison with the retention times of the reference standards.

The precision of the extraction procedure and chromatography technique were evaluated by processing as replicates in six different occasions aliquots of pooled different tissue samples containing known amounts of IVM.

Method validation

The method for the identification of IVM in tissue broilers was validated in terms of the analytical parameters of linearity, precision, accuracy, LOQ and LOD, and selectivity following the guideline for the validation of analytical methods used in residue-depletion studies from the International Cooperation on Harmonization of Technical Requirements for the Registration of Veterinary Medicinal Products (VICH 2015).

Linearity was determined using seven concentrations of IVM standard (0.5, 1, 2.5, 5, 10, 20 and 30 ng ml⁻¹) injected three times into the chromatography system.

Blank tissue samples were fortified with IVM ranging between 0.5 and 30 ng g⁻¹. IVM concentrations were determined from peak areas and the use of calibration curves obtained by running tissue samples from chickens not treated with IVM (i.e., chicken control) that were spiked with known concentrations of IVM. For tissue specimens as determined using the linear least squares regression procedure, a linear relationship existed in the calibration curve of IVM over the range of 0.5–30 µg g⁻¹ for muscle, liver, kidney and skin plus fat.

Precision and accuracy of the method were determined by the evaluation of replicates of IVM-free samples ($n = 6$) fortified with IVM at different concentrations (0.5, 5, 20, 30 ng g⁻¹). Precision was expressed as coefficient of variation (% CV). Accuracy, defined as the closeness between the experimentally measured and the true value, was determined by the differences between observed and calculated concentration results, and expressed as the relative error (% RE) (Chandran & Singh 2007).

The LOQ was calculated as the lowest IVM concentration ($n = 6$) on the standard curve that could be quantitated with precision not exceeding 20% and accuracy within 20% of nominal. The LOD was estimated by the analysis of 10 aliquots of control tissue (free of IVM). The noise of the baseline was

measured; the average and standard deviation were calculated; the LOD corresponded to three times the SD (sign/noise $\geq 3/1$).

Selectivity is the ability of the method to distinguish between the analyte being measured and other different substances that might be present in the sample being analysed. The selectivity of the method was determined by comparing the chromatograms of IVM-free tissue samples with those of each tissue fortified with IVM. The lack of interferences in the separation suggests a high specificity of the chromatographic method and good selectivity of the extraction procedure. Tissue concentrations were expressed as ng g^{-1} .

Withdrawal time

The withdrawal periods for edible tissues of chickens (muscle, liver, kidney and skin plus fat) were estimated by linear regression analysis of the log-transformed tissue concentrations and determined at the time when the upper one-sided 95% tolerance limit for the residue was below the MRL, with a confidence of 95% (EMEA 2002). IVM concentrations as a function of time found in muscle, kidney, liver and skin/fat were plotted and analysed with the program WT version 1.4 in order to recommend a period of withdrawal time for this experimental formulation.

Results

No adverse response was observed after feeding with feed supplemented with IVM at 5 mg kg^{-1} during 21 days. This method performed accurately and reproducibly over a range of $0.5\text{--}30 \text{ ng g}^{-1}$ for IVM. The linear regression equation obtained for the proposed HPLC method as calibration curve was $y = (1.6884\text{E-}5 \pm 0.0695)x + (0.2962 \pm 0.0867)$ ($r = 0.9969 \pm 0.00076$), where y is the area ratio; and x is the analyte concentration (ng ml^{-1}). The chromatographic analysis time was short and IVM was presented in $2.04 \pm 0.4 \text{ min}$, and no peaks were observed in the vicinity of IVM retention time, attesting the method's selectivity (Figure 1).

Precision and accuracy of the method were determined by evaluation of replicates of drug-free samples ($n = 6$) fortified with IVM at different concentrations ($0.5, 5, 20, 30 \text{ ng g}^{-1}$). Table 1 shows the validation parameters (R^2 , percentage

recovery, precision (% CV), accuracy (% RE) and LOD) for different tissues spiked with $0.5, 5, 20$ and 30 ng g^{-1} of IVM. The mean global percentage of recoveries were 91.8%, 92.6%, 93.9% and 91.6% in liver, kidney, muscle and fat respectively, with CVs of 4.0%, 1.5%, 2.8% and 2.9% respectively. For individual levels and the overall evaluation of the working range, satisfactory results in the region of 70–120% were obtained. Within-laboratory precisions were $\leq 20\%$ for the working range. The LOQ for each tissue assayed was 0.5 ng g^{-1} , the lowest concentration of IVM residues evaluated with acceptable accuracy and precision, applying the complete analytical method here presented. The calculated LODs are presented in Table 1.

The validation method used here indicated that the analytical method used to extract, derivatise and quantify IVM in chicken tissues by chromatographic analysis using a fluorescence detector was appropriate.

IVM tissue concentrations

Table 2 shows the mean \pm SD tissue concentrations of IVM in muscle, kidney, liver and skin plus fat on different days after the end of treatment. The residue levels were low, with the highest concentration measured between the first and second days post-treatment in all tissues. The tissue with the highest concentration was liver, followed by skin/fat, kidney, and muscle. The higher concentrations were found in liver tissue, which is logical since IVM is a molecule that undergoes extensive hepatic metabolism, mainly by hydroxylation processes.

Considering that IVM was administered with food, concentrations found in different tested tissues were highly variable.

Linear regression analysis of the logarithmic transformed data can be considered for the calculation of the withdrawal periods. Using this approach, the withdrawal time is determined as the time when the one-sided 95% upper tolerance limit of the regression line with a 95% confidence level is below the MRL. According to the residual concentrations found, optimal withdrawal times for edible tissues were 12 days for liver, 8 days for skin/fat and 10 days for kidney (Figure 2). In muscle tissue the concentrations found were always below the MRL established (30 ng g^{-1}), that way the assay did not give any withdrawal time.

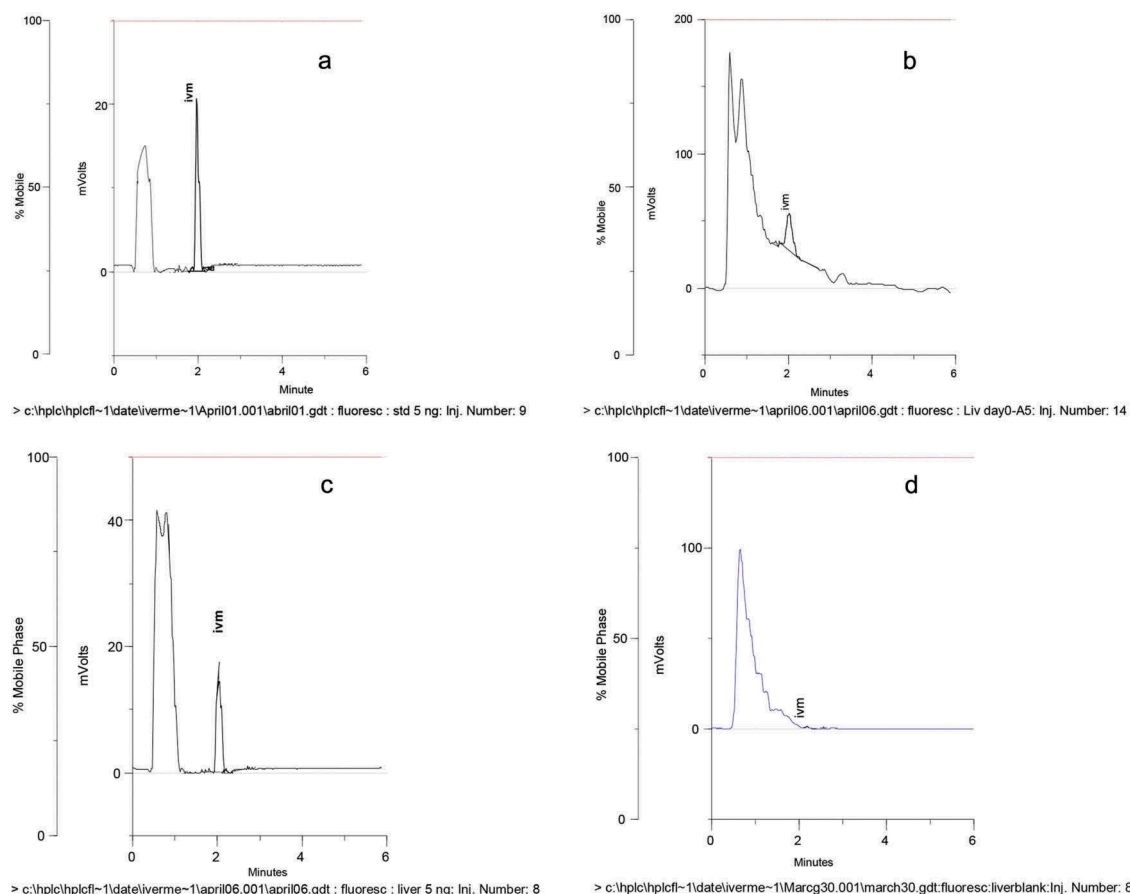


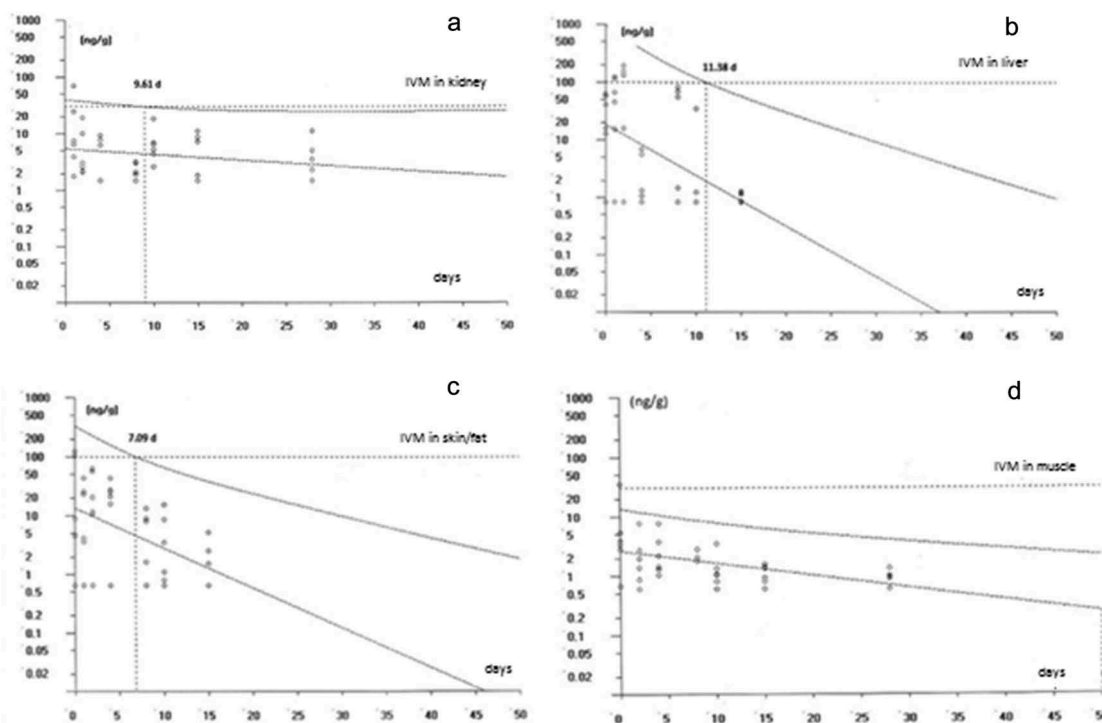
Figure 1. (colour online) Chromatograms of 5 ng ml⁻¹ IVM standard (A), liver sample obtained at 0 day post-treatment to animal 5 (B), liver tissue fortified with 5 ng g⁻¹ of IVM (C) and liver control (blank).

Table 1. Validation parameters for the determination of ivermectin residues in chicken tissues.

Parameter	Chicken tissue			
	Liver	Kidney	Muscle	Skin/fat
R^2	0.9969	0.9985	0.9957	0.9970
0.5 ng g^{-1} ($n = 6$)				
Recovery (%) \pm SD	95.6 \pm 5.1	98.6 \pm 0.2	101.1 \pm 3.0	96.2 \pm 2.2
Precision (% CV)	5.3	0.2	3.0	2.6
Accuracy (% RE) \pm SD	-4.4 \pm 5.1	-1.4 \pm 0.2	1.1 \pm 3.0	-3.8 \pm 2.2
5.0 ng g^{-1} ($n = 6$)				
Recovery (%) \pm SD	88.5 \pm 2.7	92.4 \pm 3.1	89.9 \pm 5.4	94.3 \pm 3.6
Precision (% CV)	3.0	3.4	6.0	3.9
Accuracy (% RE) \pm SD	-11.5 \pm 2.7	-7.6 \pm 3.1	-10.0 \pm 5.4	-5.7 \pm 3.6
20 ng g^{-1} ($n = 6$)				
Recovery (%) \pm SD	87.5 \pm 2.6	81.3 \pm 1.5	85.2 \pm 0.6	83.7 \pm 1.9
Precision (% CV)	3.0	1.87	0.6	2.3
Accuracy (% RE) \pm SD	-12.6 \pm 2.6	-18.7 \pm 1.5	-14.9 \pm 0.6	-16.3 \pm 1.9
30 ng g^{-1} ($n = 6$)				
Recovery (%) \pm SD	95.5 \pm 4.6	98.1 \pm 0.7	99.5 \pm 1.4	93.4 \pm 2.9
Precision (% CV)	4.8	0.7	1.4	3.1
Accuracy (% RE) \pm SD	-4.5 \pm 4.6	-1.9 \pm 0.7	-0.5 \pm 1.4	-6.6 \pm 2.9
<i>Overall evaluation over the working range</i>				
Recovery (%) \pm SD ($n = 24$)	91.8 \pm 4.4	92.6 \pm 8.1	93.9 \pm 7.6	91.6 \pm 5.6
Precision (% CV)	4.0	1.5	2.8	2.9
Accuracy (% RE) \pm SD	-7.0 \pm 3.3	-5.1 \pm 4.0	-2.6 \pm 5.0	-5.0 \pm 1.4
LOD (ng g ⁻¹)	0.11	0.07	0.05	0.09

Table 2. Mean \pm SD of ivermectin (ng g^{-1}) concentrations measured in the depletion study in chickens after oral administration of IVM during 21 days with the food.

Days post-treatment	Kidney \pm SD	Liver \pm SD	Skin/fat \pm SD	Muscle \pm SD
0	9.57 \pm 3.58	38.32 \pm 13.33	7.91 \pm 3.73	8.63 \pm 3.18
1	18.92 \pm 5.88	73.54 \pm 36.40	5.86 \pm 3.12	4.49 \pm 2.52
2	6.55 \pm 2.93	124.10 \pm 65.54	49.41 \pm 29.72	2.55 \pm 2.68
4	5.89 \pm 3.79	3.76 \pm 3.03	14.06 \pm 11.76	2.94 \pm 2.61
8	2.28 \pm 1.87	52.88 \pm 26.11	32.37 \pm 15.01	2.29 \pm 0.47
10	7.38 \pm 3.67	18.19 \pm 13.95	26.71 \pm 10.17	1.53 \pm 1.09
15	7.14 \pm 3.86	1.12 \pm 0.19	3.11 \pm 1.88	1.21 \pm 0.34
21	7.06 \pm 2.62	3.81 \pm 2.65	2.19 \pm 1.38	0.93 \pm 0.34
28	4.70 \pm 1.90	1.07 \pm 0.05	2.01 \pm 1.05	0.88 \pm 0.30

**Figure 2.** Withdrawal times of IVM calculated by linear regression analysis for kidney (a), liver (b), skin/fat (c) and muscle (d) (logarithmic transformed data).

Discussion and conclusions

Regarding the analytical methodology, for the working range evaluated, recovery and precision are according to the performance criteria for considering a quantitative method suitable for the determination of veterinary drug residues in foods when the concentration is between 1 and 100 $\mu\text{g g}^{-1}$ (FAO & OMS 2009).

IVM is used in different animals (cattle, sheep, goats, pigs, horses) at dose rates of 100–500 $\mu\text{g kg}^{-1}$ by subcutaneous, topical or oral routes, as a single-dose treatment only. Furthermore, it has also been studied in other animal species such as wild ruminants (reindeer, deer,

camels and American bison) for which IVM extra-label use has been reported (Gonzalez-Canga et al. 2012; Moreno et al. 2015).

In poultry, anti-parasitic compounds are used extensively for disease prevention and treatment. Sharma et al. (1990) evaluated the IVM efficacy against *Ascaridia galli* infection in chickens at a dose of 300 $\mu\text{g kg}^{-1}$ subcutaneously, finding an efficacy between 90% and 95% against immature and adult worms. Moreover, they found that the treated birds also had a better growth rate than the untreated control chickens.

In addition to controlling parasites effectively, some authors found an immunostimulatory effect

in broilers at a high-rate dose of IVM (5 mg kg^{-1}) (López-Olvera et al. 2006; Omer et al. 2012). Omer et al. (2012) treated 20-day-old chickens with IVM at dosage rates of 0.15, 0.3, 1, 3 and 5 mg kg^{-1} body weight. In another study carried out by Khayatnouri et al. (2011), the effect of IVM pour-on administration against *Heterakis gallinarum* infestation was evaluated at a dose of $500 \mu\text{g kg}^{-1}$. They obtained a reduction in egg count exceeding 98%, concluding that IVM can be used in antiparasitic programmes in poultry.

However, the available information reporting tissue residue profiles after IVM treatment is scarce in poultry compared with other species (Goetting et al. 2011; Moreno et al. 2015). Edible tissues containing veterinary drug residues can pose risks to human health, including direct toxic effects, allergic reactions and increased parasites resistance (Ōmura & Crump 2014).

Macrolide endectocides, such as IVM, are lipophilic compounds, so high concentrations will be found in edible tissues, particularly in those with a high fat content, where they can persist for prolonged periods (Canga et al. 2009). IVM is not highly metabolised and excretion is primarily via the faeces (Canga et al. 2009).

When IVM is administered to laying hens, residues are preferentially deposited in the egg yolk (Keukens et al. 2000; Moreno et al. 2015) and can be found in eggs laid for several days following cessation of treatment. Moreno et al. (2015) treated laying hens with IVM administered daily in water at $400 \mu\text{g kg}^{-1}$ dose for a 5-day period. They quantified IVM residues for a longer time in skin/fat until 15 days post-treatment, in the case of muscle and liver until 7 days after the end of treatment, and IVM kidney residues were measured only 3 days post-treatment. In contrast, Bennett and Cheng (2012) treated adult pigeons brooding squab with IVM in drinking water ($3.3 \mu\text{g ml}^{-1}$) for 3 days and found high IVM residue concentrations in both liver (58.5 ng g^{-1}) and breast muscle (43.1 ng g^{-1}) at the end of treatment. In the present study, we found measurable concentrations below the MRL in all tissues until 28 days post-treatment. These differences can be explained by the vehicle used to solubilise the IVM. Moreno et al. (2015) and Bennett and Cheng (2012) used drinking water and we used initiator chickens' feed. The main drinking water

medication disadvantages are related to the several factors that influence individual animal water intake, including biological (body weight, age), environmental (lighting period, environmental temperature) and management factors (composition of the diet).

Our results do not match those of Miller (1990) who administered IVM to chickens with a diet of $2 \mu\text{g g}^{-1}$ of food for 5 weeks and found no residues of IVM in their livers, so this author did not establish a withdrawal period. The study performed by Miller was mainly about IVM efficacy against *Alphitobius diaperinus*, not about tissue profile depletion of IVM, where the experimental animals were sacrificed at the end of the treatment (5 weeks), therefore IVM residual levels in the liver were determined only at that sampling time.

In the European Union, MRLs of 100 (fat and liver) and 30 ng g^{-1} (for kidney and muscle) are established for IVM in all mammalian food-producing species. Withdrawal periods of 35, 28 and 18 days are recommended after IVM administration by the subcutaneous route in cattle, sheep and pigs, respectively. In some Latin American countries there are some IVM commercial formulations for use in the poultry industry. They are usually administered in water or food for 2, 3, 5 or 21 days. Withdrawal times are recommended in some of these formulations (2 or 7 days for meat and 0 days for eggs). According to European Union regulations, chickens fed for 21 days with this type of supplement containing IVM (5 mg kg^{-1}) would be suitable for human consumption at 12 days post-treatment, i.e., at 33 days of life in this case. Considering that broiler chickens aged 45–54 days old are destined for the market, it can be stated that with the dosage scheme proposed in our study the chickens would be suitable for human consumption. These results would suggest that the IVM withdrawal period for growing chickens could be shorter than that of mammals, but pharmacokinetic studies need to be performed to test this hypothesis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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