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ORIGINAL ARTICLE

Oral and topical extra-label administration of fipronil to laying hens: Assessment of the egg residue patterns

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Abstract

This experimental work reproduces the fipronil extra-label administration performed by producers in laying hens. The scientific goal was to characterize the residual concentrations in eggs from treated hens and suggest the withdrawal periods that should be respected to avoid risk for consumers. Thirty-four laying hens were allocated into two groups: Group A was treated with fipronil in feed, two single doses of 1 mg kg^{-1} day^{-1}; Group B was administered a single dose of 1 mg kg^{-1} by the topical route. Fipronil egg residues were quantified by HPLC-MS/MS. Fipronil and its sulphone metabolite (fipronil-SO_2) were measured in egg after both treatments. The highest egg residual profile was always for fipronil-SO_2. Mean maximum egg concentrations (C_{max}) of 228.5 ± 79.8 ng/g (fipronil) and 1,849 ± 867 ng/g (fipronil-SO_2) were found after fipronil administration in feed. The lowest residual levels were quantified after the topical treatment with C_{max} of 27.1 ± 4.9 and 163 ± 26 ng/g for fipronil and fipronil-SO_2. Mean fipronil marker residues and established MRLs allowed calculating the withdrawal periods, the shortest being 74 days after topical administration. Such a long withdrawal period is difficult to meet in egg production systems. Thus, the extra-label use of fipronil in laying hens should not be recommended under any circumstances.

KEYWORDS
egg residues, extra-label use, fipronil, laying hens

1 | INTRODUCTION

Fipronil is a phenylpyrazole pesticide widely used to control many agricultural and domestic pests. It has broad-spectrum activity against fleas, ticks, mites and lice (Dryden, 2015). It is available to treat fleas and ticks in cats and dogs; as an insect bait against cockroaches, ants and termites; and widely applied in agriculture for soil treatment, seed coating and crop protection (Gupta & Milatovic, 2014).

Fipronil is not licenced to treat laying hens. However, some international news in 2017 revealed that high fipronil residue levels were detected in eggs after poultry was accidentally exposed to this drug in the Netherlands and other EU countries (Polet & Smith, 2017). In this case, the exposure was unintended; however, in others, the reason for which fipronil egg residues have been related to poultry is the treatment of a mite, specifically the red mite *Dermanyssus gallinae*.

D. gallinae is the most significant ectoparasite pest in poultry (Chauve, 1998). It is found in all production systems, including organic, intensive, enriched cage or barn (Sparagano et al., 2014). It sucks laying hens blood at night and then hides in the crevices and litter of poultry houses during the day. Infested laying hens can develop anaemia (Cosoroaba, 2001; Kilpinen, 2005), decrease their feed intake, egg production, egg quality (shell thinning, spotting) and weight gain, and in severe cases, they may die (Sigognault Flochlay

et al., 2017). *D. gallinae* can also be involved in the transmission of numerous poultry pathogens, including zoonotic pathogens such as *Salmonella enteritidis* (Mul, 2009; Sommer et al., 2016; Valiente Moro et al., 2009, 2010).

Consequently, the economic impact of this pest on the poultry sector is considerable. In Europe, the annual cost of the red mite infestation was estimated at €231 million for productivity losses (Sigognault Flochlay et al., 2017; Van Emous, 2017).

The control of *D. gallinae* is a problem because very few drugs have been approved to treat ectoparasites in laying hens. Only two active drugs, phoxim and the more recently fluralaner, are licenced for red mite treatment of poultry in some countries (European Commission, 2017; Prohaczik et al., 2017). The lack of accessible drugs to treat the red mite in laying hens has led to illegal extra-label use of acaricides, such as fipronil, in many countries (Marmulak et al., 2015; Reich & Triacchini, 2018). The extra-label use of different compounds is allowed in particular situations, but this practice is very well regulated with laws (Comyn, 2003; Directive EC, 2001; FDA, 2019; Government of Canada, 2014) in order to prevent the appearance of illegal residues in human food, such as meat, eggs, milk and other products. Consequently, the rules for legitimate extra-label use of drugs in food animals, including poultry, are more restrictive than those for strictly companion animals (Spenser, 2004).

Although extra-label use could be an alternative to control the red mite in poultry, the problem with fipronil is that it has not been classified as an 'allowed substance' for food-producing animals in most countries. In the EU, fipronil was only authorized as an insecticide in plant protection (Regulation EC, 2009). It could never have been a treatment option for food-producing animals following the cascade system (Directive EC, 2001). Additionally, fipronil is an EPA-regulated pesticide, and extra-label use of this product is expressly prohibited in the United States, including all poultry, either in large commercial or small backyard flocks (Stafford et al., 2018).

Despite the aforementioned restrictions, the strong need for accessible drugs to control the red mite in poultry has led to the illegal use of fipronil in many countries (Reich & Triacchini, 2018).

In Argentina, there is an average population of 139 million industrial birds, including broilers (71%), laying hens (27%), breeding parents (1%) and grandparents (1%) (SENASA, 2020). The poultry sector is very important because of its contribution to the basic food basket, with a great economic impact on both local consumption and exports. The fipronil extra-label use is also evident, as a fipronil pour-on formulation licenced for cattle has been used for this purpose in egg producing farms for local consumption. In this context, in 2015, this research subject was began in our laboratory, aiming to answer several questions concerning the kind of treatment performed, the doses used, the occurrence of residues in eggs, among others. From conversations with the poultry producers, it was found out the type of extra-label fipronil administration that they were 'hypothetically' using in laying hens. At the same time, some egg samples were taken from a farm, and it did indeed find fipronil residues in egg (Canton et al., 2018). In this context, the aims of the current experimental work were as follows: 1. to reproduce the extra-label administrations performed by producers in laying hens in a 'controlled experimental' way; 2. to determine the occurrence of fipronil residues in eggs after these administrations; 3. to suggest a withdrawal period for fipronil extra-label use in hens.

2 | MATERIALS AND METHODS

2.1 | Reagents and chemicals

Pure reference standards (99% purity) of fipronil and fipronil-SO_2 were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada). The acetonitrile solvent used during the extraction and drug analysis was HPLC grade and purchased from Sintorgan S.A. (Buenos Aires, Argentina). Water was double distilled and deionized using a water purification system (Simplicity®; Millipore, Sao Paulo, Brazil).

2.2 | Fipronil treatments

2.2.1 | Fipronil feed medication (Group A)

Fipronil (Ectoline®, Merial Argentina S.A., fipronil 1%) was administered in feed. We considered the following aspects for feed medication. The administered dose was 1 mg/kg, defined according to the extra-label practice used in avian production, whereby 2 L of Ectoline (1%) is mixed with 1000 kg of feed (20 g of fipronil/1000 kg of feed = 20 μg/g). The daily feed consumption of 150 g/day/hen and the average weight of the animals (2.9 kg) were taken into account. The formulation volume to be administered was mixed in a diffuser containing 50 ml of sunflower oil and stirred manually to ensure a homogeneous mixture. This mixture was dispersed over the food on a tray administered in batches and mixed mechanically (by mixer) for 15 min to guarantee a uniform mix. The medicated feed was prepared just before administration. As fipronil concentration in feed was not checked, it was assumed that the form and time of mixing guarantee the homogeneous drug distribution in feed. However, some lack of uniformity in drug mixing could have contributed to the variability of fipronil concentrations quantified in egg.

2.2.2 | Topical fipronil (Group B)

Fipronil (Ectoline®, Merial Argentina S.A., fopronil 1%) was administered topically at the dose of 1 mg/kg. According to the average weight of the animals (2.9 kg), 0.3 ml of the pure formulation was administered. Imitating the extra-label practice in poultry production, the volume was deposited by two or three drops on the caudal half of the animal's dorsal line.

2.3 | Experimental design

All the experiments were carried out at "Escuela de Educación Secundaria Agraria No1, Dr. Ramón Santamarina" (Tandil, Buenos Aires, Argentina), following ethical guidelines of the animal welfare committee of the Facultad de Medicinina Veterinaria, Universidad Nacional del Centro de la Provincia de Buenos Aires (Act 087/02). Thirty-four (34) *Plymouth Rock Barrada* adult laying hens (10–12 months old, 2.9 ± 0.4 kg body weight) were involved in the current experiment. The hens were housed and acclimated for 10 days with water and balanced commercial food (Metrive®) *ad libitum*. They did not receive any medication before the experiments. They were allocated into two groups (17 animals each) and treated as follows: Group A: Fipronil feed medication. Hens were treated with fipronil by the oral route in feed (1 mg fipronil/kg) prepared as described above. Two administrations were carried out, the first one at day 1 and the second one at day 7. Group B: Topical route. The hens were administered fipronil by the topical route, in one single dose (1 mg fipronil/kg) as described previously. Eggs produced daily from the first administration until 35 days post-first treatment were collected. Group and sampling day were written on the shell for future identification. In the laboratory, the eggs were opened, and the yolk and white were mixed (intense manual stirring with a spoon for three min) and stored in a plastic tube at −18°C until further analysis by HPLC-MS/MS.

2.4 | Egg analysis

Experimental or fortified egg samples were processed and analysed by HPLC-MS/MS following a methodology developed in our laboratory. Total egg samples (0.5 g) were extracted by the addition of 1 ml cold acetonitrile under a high-speed vortexing shaker for 10 min. After sonication for 10 min, samples were centrifuged (4000 rpm, 10 min, 4°C). The clear supernatant was added with 2 ml of water and transferred to C_{18} cartridges (100 mg/ml Strata C18-T, Phenomenex, CA, USA) using a manifold vacuum (Baker spe-24G). The cartridges were previously conditioned with 1 ml of methanol (HPLC grade), followed by 1 ml of water (HPLC grade). All samples were applied and then sequentially washed with 1 ml of water, 1 ml of methanol/water (1:4) and 1 ml of hexane, dried with air for 2 min and eluted with 2 ml of acetonitrile (HPLC grade). The eluted volume was evaporated to dryness at 60°C in a vacuum concentrator (Speed-Vac, Savant, Los Angeles, CA, USA). The dry residue was dissolved in acetonitrile:water (60:40) 2 ml by shaking (10 min) and sonication (10 min). The samples were filtered with 0.22 µm nylon filters and 5 µl were injected into the chromatographic system.

2.5 | HPLC-MS/MS system and chromatographic condition

Samples were analysed by HPLC-MS/MS using equipment from Shimadzu (Kyoto, Japan). The equipment was composed of two HPLC-LC-20AD Prominence pumps, a SIL-20AC HT Prominence injector, a CTO-20AC Prominence column oven and an LCMS-8050 triple quadrupole mass spectrometer. A Shim-pack HR-ODS C_{18} analytical column (15 cm · 3 mm internal diameter, 2.6 mm particle size) at 40°C in the column oven was used for separation. Water (A) and ACN (B) were used as the mobile phase at 0.4 ml·min^{-1} with the following gradient programme. Initially, the mobile phase (B) was 60% (0 min), increased linearly to 80% B (2 min), followed by a linear increase to 90% B (5 min) and decreased to 60% B (5.5 min), which was held until the end of the process for 7 min. The injection volume was 5 µl.

The analysis was performed in the negative ion electrospray ionization mode (ESI). Monitoring was done in multiple reaction monitoring, with a dwell time of 50 ms. Two transitions were followed for each molecule, the first one being the quantifier and the second one the qualifier. The temperature parameters for the heated ESI were 300°C (interface), 250°C (desolvation line) and 400°C (heat-block). The flow rate parameters for heating (air), nebulising (N_2) and drying gas (N_2) were 10, 3 and 10 L min^{-1}, respectively. The optimization procedure for determining individual compounds' MRM transitions, the best quantifier, qualifier ion and collision energies (eV) was made by direct injections (0.1 mg·mL^{-1}) in the Mass Spectrometer. Table 1 lists the retention time, collision energy, and the quantification and confirmation ions monitored for fipronil and fipronil-SO_2 analytes.

2.6 | Method validation

A complete validation of the analytical procedures for the extraction and quantification of fipronil and fipronil-SO_2 in egg was performed before the analysis of the experimental samples. Stock and working solutions of both standards in methanol were prepared. Parameters such as linearity, recovery, limit of detection (LOD), limit of quantification (LOQ), stability and matrix effect were investigated under the optimized extraction conditions. The linearity was tested by constructing calibration curves with blank egg samples fortified with

TABLE 1 HPLC-MS/MS parameters for fipronil and its fipronil-SO_2 metabolite: retention time, ions monitored with the multiple reaction monitoring mode (MRM), ionization mode and collision energy

	Retention time	Ionization mode (polarity)	Precursor (m/z)	Product ion (m/z)[a]	Collision energy (V)	Product ion (m/z)[b]	Collision energy (V)
Fipronil	5.30	ESI(−)	434.85	329.9	16	249.9	25
Fipronil-SO_2	5.77	ESI(−)	450.80	414.9	16	281.95	25

[a]Used for quantitation.

[b]Used for confirmation.

fipronil and fipronil-SO_2 at two ranges of calibration: 10–100 ng/g and 100–1000 ng/g. The data were analysed for linearity using the least-squares regression method, and the Run Test and ANOVA to determine whether the data differed from a straight line. The linear regression lines for fipronil and fipronil-SO_2 showed correlation coefficients ≥0.998. Mean absolute recovery percentages ranged between 78.4 and 85.8%. Precision and accuracy (intra-day and inter-day) were determined by the evaluation of replicates (n = 6) of drug-free egg samples fortified with each compound at three different concentrations (40, 100 and 1000 ng/g). The evaluation of the intra-day precision involved six (n = 6) measurements of the egg samples at the three different concentrations within a single run. Precision was expressed as the coefficient of variation (%CV). The inter-assay precision of the analytical procedures obtained after the analysis of fipronil and fipronil-SO_2 on different working days showed CV between 1.6 and 9.2%. Accuracy of the method was measured by the differences between observed and calculated concentration results obtained inter-day (6 consecutive working days) and expressed as the relative error (%RE) with values between 3.6 and 17.6%. The long-term stability of each compound was tested with blank egg samples fortified at 30 ng/g or 1000 ng/g and stored at −20 °C. Samples (n = 3) were analysed at 0 and 90 days post-freezing. Stability given by CV after analyses was between 4.4 and 17.6%. The LOQ was defined as the lowest drug concentration on the standard curve that could be quantitated (n = 6) with a precision not exceeding 20% and accuracy within 20% of nominal (Snyder et al., 1997). The LOQ values were established at 10 ng/g for both molecules. Matrix effect (%) was calculated by comparing the calibration curve slopes (CS) within the matrix-matched standards (egg) and solvent standards using the following equation:

$$(\%) = \frac{(CS - solventstandardCS)}{SolventstandardCS} x100$$

The matrix effect values were −9.2 and −2.1 for fipronil and fipronil-SO_2, respectively.

2.7 | Withdrawal period calculation

The marker residue concentrations (fipronil+fipronil-SO_2) measured in egg were analysed, and a recommended withdrawal period was estimated in egg after both fipronil administrations to laying hens. The withdrawal periods were calculated using the results of a linear regression analysis of the log residual concentrations *vs*. time of the terminal elimination phase. As there are no MRLs for the use of fipronil as a medicine in laying hens, similar to reported works (Reich & Triacchini, 2018) the MRLs established for this species associated with the use of fipronil as a pesticide were taken as references. The withdrawal period was established at the time when the upper one-sided tolerance limit with a given confidence interval (95%) was below the different MRLs/tolerance considered 5 ng/g (Commission Regulation EU, 2019); 20 ng/g (FAO/WHO, 2019); or 30 ng/g (Code of Federal Regulations, 2000). If this time point did not make up a full day, the withdrawal period was rounded up to the next day. The calculations were done using the 'Melk WTM 1.4' withdrawal-time calculation computer software (http://www.ema.europe.eu).

2.8 | Pharmacokinetic and statistical analysis

The peak concentration (C_{max}) and time to peak concentration (T_{max}) were read from the plotted concentration–time curve for each analyte. The area under the concentration–time curve (AUC) for fipronil/fironil-SO_2 residues in egg was calculated by the trapezoidal rule (Gibaldi & Perrier, 1982) using the PK Solution 2.0 software (Summit 10 Research Services, CO, USA). This parameter and the concentration data are reported as mean ± SD. Statistical comparison was carried out by 'Student´s t test' using the Instat 3.0 Software (Graph Pad Software, San Diego, CA, USA). A value of $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Administration of fipronil-medicated feed

Egg residues were detected after fipronil in feed administration to laying hens at 1 mg/kg (two doses) at all sampling times (from day 3 to 35). Fipronil parent drug residues were quantified in egg from day 3 to 23, reaching a maximum at day 12 (C_{max}: 228.5 ± 79.8 ng/g) (Figure 1). The highest egg residual profile was measured for fipronil-SO_2 (Figure 1). High egg residues of this molecule were quantified from day 3 up to the last sampling time. Although a C_{max} (1,849 ± 867 ng/g) was quantified at day 22, it was not a typical peak concentration, as fipronil-SO_2 residues increased from day 3 to 14,

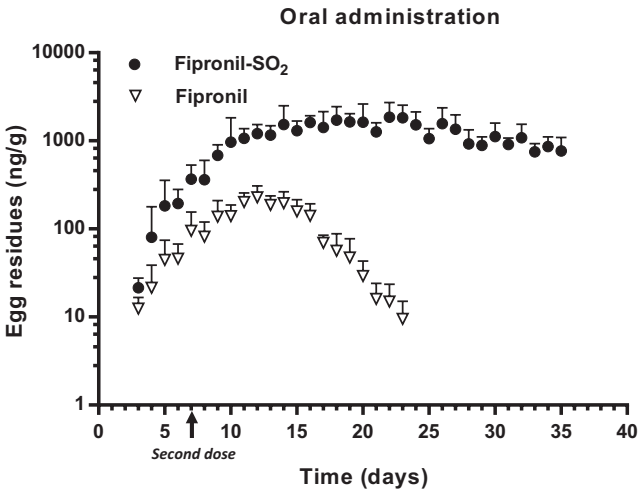


FIGURE 1 Mean (±*SD*) fipronil and fipronil-SO_2 (sulphone metabolite) egg residue profile *vs*. time obtained after fipronil administration in feed (two single doses of 1 mg kg^{-1} day^{-1} at 1 and 7 days) to laying hens

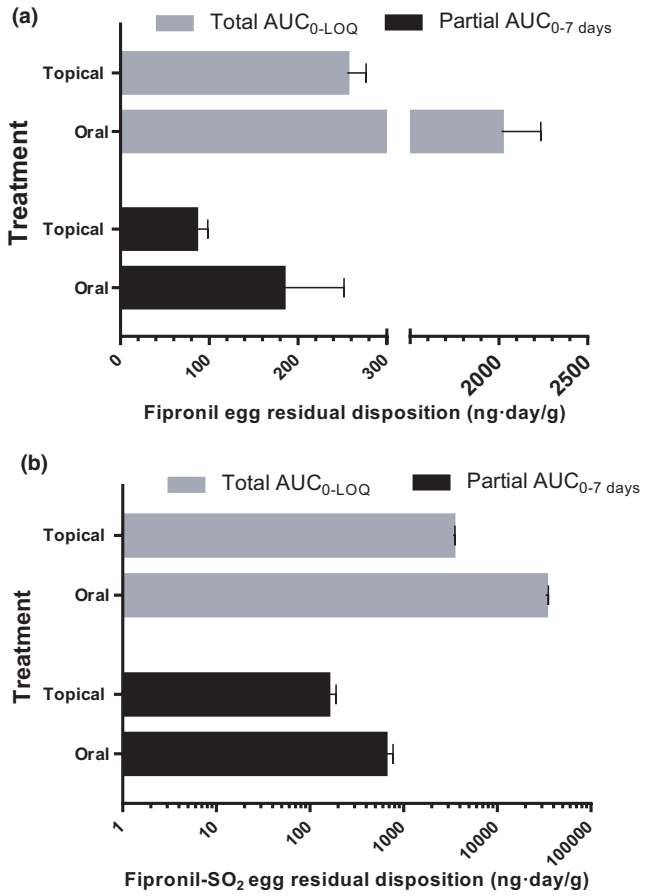


FIGURE 2 Egg residual concentrations as mean AUC (area under the curve) (±*SD*) (ng·day/g) for fipronil (a) and fipronil-SO_2 (sulphone metabolite) (b) after fipronil administration in feed (two single doses of 1 mg kg^{-1} day^{-1} at 1 and 7 days) or topical (a single dose of 1 mg kg^{-1} day^{-1} at day 1) administration to laying hens. Two AUC values are shown for each molecule and each treatment route: total AUC ($AUC_{0\text{-}LOQ}$) (including two doses) and partial AUC ($AUC_{0\text{-}7\ days}$) (including one dose)

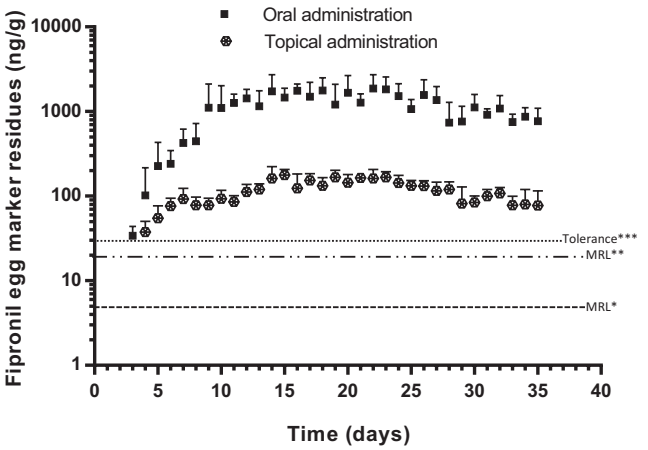


FIGURE 3 Mean (±*SD*) fipronil egg marker residues (sum of fipronil parent drug plus fipronil-SO_2 (sulphone metabolite)) profile *vs.* time obtained after fipronil administration in feed (two single doses of 1 mg kg^{-1} in feed at days 1 and 7) and topical (one single dose of mg kg^{-1} day^{-1} at day 1) administration to laying hens. *5 ng/g (Commission Regulation EU, 2019); **20 ng/g (FAO/WHO, 2019); ***30 ng/g (tolerance) (Code of Federal Regulations, 2000)

then remained in a 'plateau' until day 24, and afterwards began to decrease very slowly.

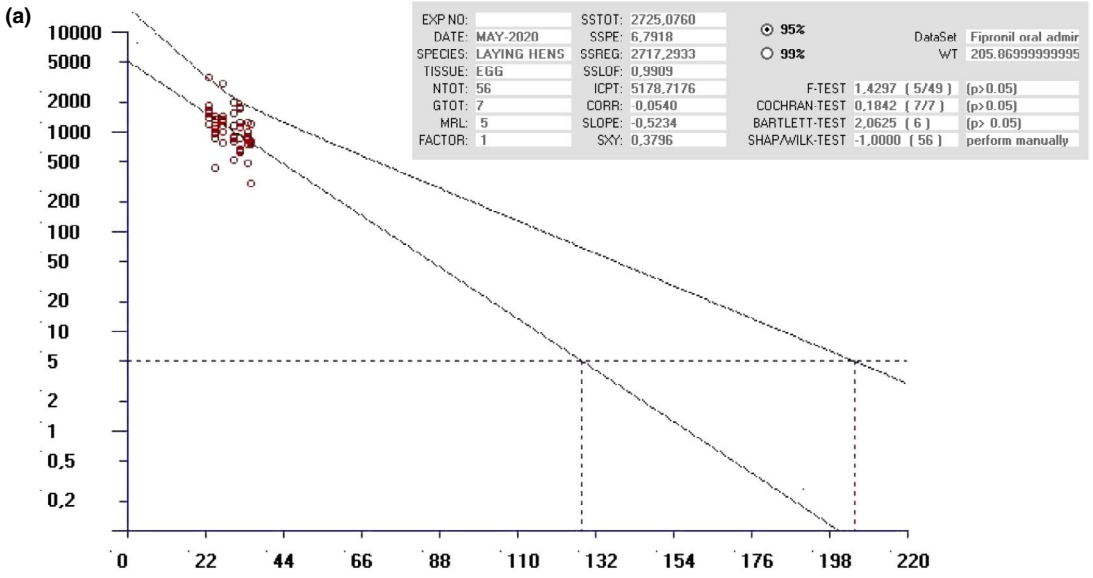
Figure 2 shows the egg fipronil concentrations after its administration in feed estimated as partial ($AUC_{0\text{-}7\ days}$) or total ($AUC_{0\text{-}LOQ}$) area under the curve. Figure 3 shows egg marker residues (fipronil+fipronil-SO_2) after fipronil administration in feed to laying hens. The concentration *vs.* time curve profile of the marker residue was represented mostly by fipronil-SO_2, as higher concentrations of this metabolite were quantified in eggs, compared to the parent fipronil. Peak egg concentration (C_{max}) of egg marker residue C_{max} (1864 ± 866 ng/g) at 22 days was similar to fipronil-SO_2 $C_{max.}$ (1849 ± 867 ng/g). Regarding fipronil maximum residue limits (MRLs) allowed in egg—5 ng/g (Commission Regulation EU, 2019); 20 ng/g (FAO/WHO, 2019); or 30 ng/g (tolerance) (Code of Federal Regulations, 2000)—the fipronil egg marker residues were well above these MRLs from day 3 in all samples taken after oral treatment. To understand the magnitude of non-compliant residue levels, the ratios between fipronil egg marker residues at each sampling time and MRLs (5; 20 or 30 ng/g) were calculated. The ratio values were in the range of 7 to 373 (MRL: 5 ng/g); 2 to 93 (MRL: 20 ng/g); and 1(Day 3 only) to 62 (tolerance: 30 ng/g). Withdrawal period calculations using the results of a linear regression analysis of the log residual concentrations *vs.* time of the terminal elimination phase after fipronil administration in feed are shown in Figure 4. The suggested withdrawal periods are reported in Table 2.

3.2 | Fipronil topical administration

After fipronil single topical administration (1 mg/kg) to laying hens, significant egg residues were quantified (Figure 5). Low fipronil residues were quantified in egg over the LOQ from day 4 to 16, reaching the maximum at day 7 (C_{max}: 27.1 ± 4.9 ng/g). The highest egg residual profile after topical administration corresponded to the fipronil-SO_2 metabolite, which was quantified from day 4 until the last sampling time. Although fipronil-SO_2 residual levels observed after the topical route were much lower than those observed after the administration in feed, the curve profiles had a similar shape. After topical treatment, fipronil-SO_2 residues also increased from day 4 to 14, and then egg concentrations remained in a plateau until day 24, decreasing very slowly afterwards. The C_{max} was 163 ± 26 ng/g quantified at day 14.

Figure 2 shows the egg fipronil concentration after its topical administration estimated as partial ($AUC_{0\text{-}7\ days}$) or total ($AUC_{0\text{-}LOQ}$) area under the curve. Figure 3 shows egg marker residues (fipronil+fipronil-SO_2) after fipronil topical administration to laying hens. As previously described, as higher concentrations of fipronil-SO_2 were observed in eggs, the marker residue curve profile was determined by this metabolite. The marker residue C_{max}

(a)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TISSUE: EGG
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GTOT: 7
MRL: 5
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SSPE: 6,7918
SSREG: 2717,2933
SSLOF: 0,9909
ICPT: 5178,7176
CORR: -0,0540
SLOPE: -0,5234
SXY: 0,3796
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99%
DataSet Fipronil oral admir
WT 205.86999999995
F-TEST 1,4297 (5/49) (p>0.05)
COCHRAN-TEST 0,1842 (7/7) (p>0.05)
BARTLETT-TEST 2,0625 (6) (p> 0.05)
SHAPAWILK-TEST -1,0000 (56) perform manually
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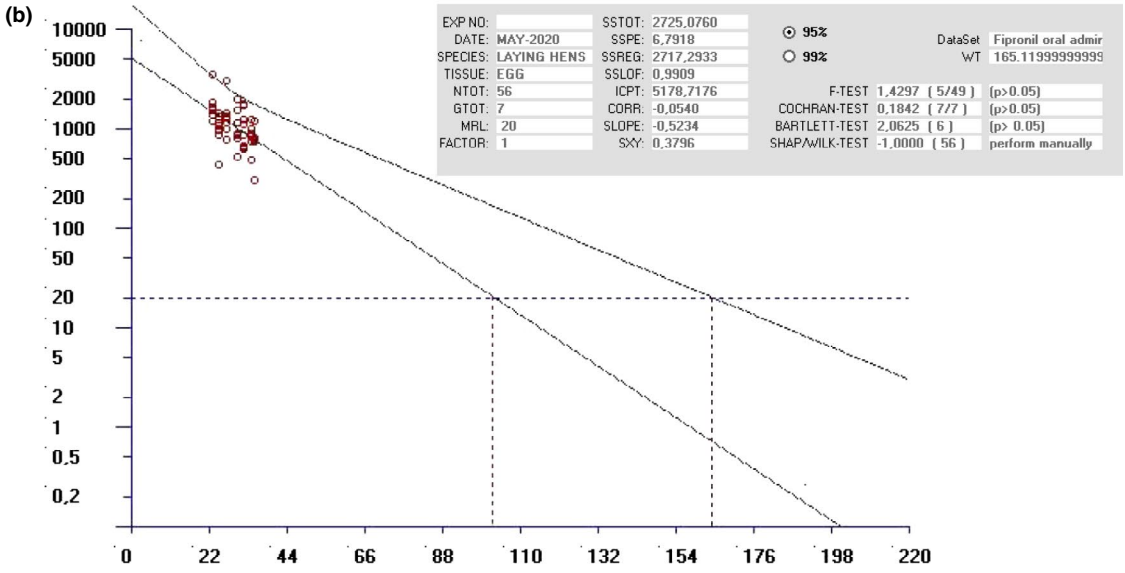
(b)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SSREG: 2717,2933
SSLOF: 0,9909
ICPT: 5178,7176
CORR: -0,0540
SLOPE: -0,5234
SXY: 0,3796
95%
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DataSet Fipronil oral admir
WT 165.11999999995
F-TEST 1,4297 (5/49) (p>0.05)
COCHRAN-TEST 0,1842 (7/7) (p>0.05)
BARTLETT-TEST 2,0625 (6) (p> 0.05)
SHAPAWILK-TEST -1,0000 (56) perform manually
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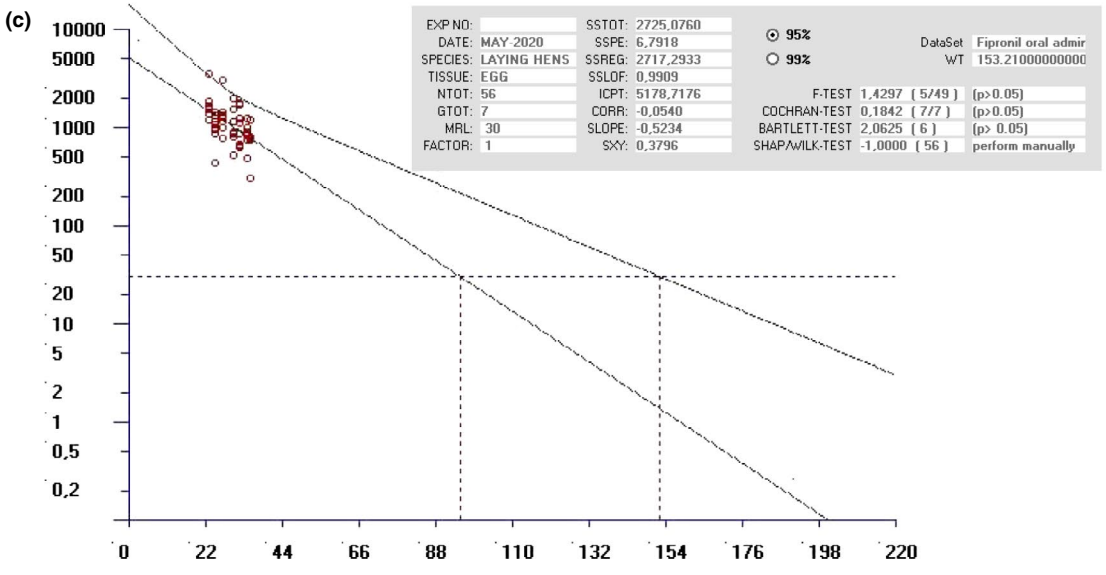
(c)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ICPT: 5178,7176
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SLOPE: -0,5234
SXY: 0,3796
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99%
DataSet Fipronil oral admir
WT 153.21000000000
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BARTLETT-TEST 2,0625 (6) (p> 0.05)
SHAPAWILK-TEST -1,0000 (56) perform manually
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FIGURE 4 Withdrawal period calculations using the results of a linear regression analysis of the log residual concentrations *vs*. time of the terminal elimination phase after fipronil administration in feed (two single doses of 1 mg kg^{-1} day^{-1} at 1 and 7 days) to laying hens.. The 'Melk WTM 1.4' withdrawal-time calculation computer software (http://www.ema.europe.eu) was used. Different MRL or tolerance values were considered: 5 ng/g (Commission Regulation EU, 2019) (a); 20 ng/g (FAO/WHO, 2019) (b); 30 ng/g (tolerance) (Code of Federal Regulations, 2000) (c)

TABLE 2 Suggested withdrawal period estimated for egg after fipronil administration in feed (two single doses of 1 mg kg^{-1} day^{-1} at 1 and 7 days) or topical (one single dose of 1 mg kg^{-1} day^{-1} at day 1) administration to laying hens

	Suggested withdrawal period (days)	
MRL/Tolerance	ORAL	TOPICAL
5 ng/g*	206	120
20 ng/g**	166	84
30 ng/g***	154	74

*5 ng/g (Commission Regulation EU, 2019);

**20 ng/g (FAO/WHO, 2019); or

***30 ng/g (Tolerance) (Code of Federal Regulations, 2000).

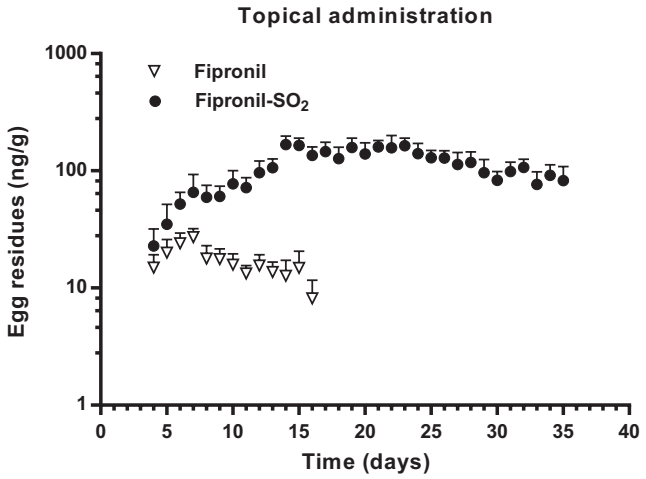


FIGURE 5 Mean (±*SD*) fipronil and fipronil-SO_2 (sulphone metabolite) egg residue profile *vs*. time obtained after fipronil topical (a single dose of 1 mg kg^{-1} at day 1) administration to laying hens

was 176 ± 30 ng/g. Although fipronil marker residues after topical administration were significantly lower than those after oral administration, they were also above the mentioned tolerance/MRLs (Code of Federal Regulations, 2000; FAO/WHO, 2019; Commission Regulation EU, 2019) from day 3 and at all sampling times, with the only exception of residue levels at day 3, which were below 20 and 30 ng/g. The ratios between fipronil marker residues at each sampling time and MRLs were in the range of 3 to 35 (MRL: 5 ng/g); 0.7(day 3 only) to 9 (MRL: 20 ng/g); or 0.5 (day 3 only) to 6 (tolerance: 30 ng/g). Withdrawal period calculations using the results of a linear regression analysis of the log residual concentrations *vs*. time of the terminal elimination phase after fipronil topical administration are shown in Figure 6. The suggested withdrawal periods are reported in Table 2.

4 | DISCUSSION

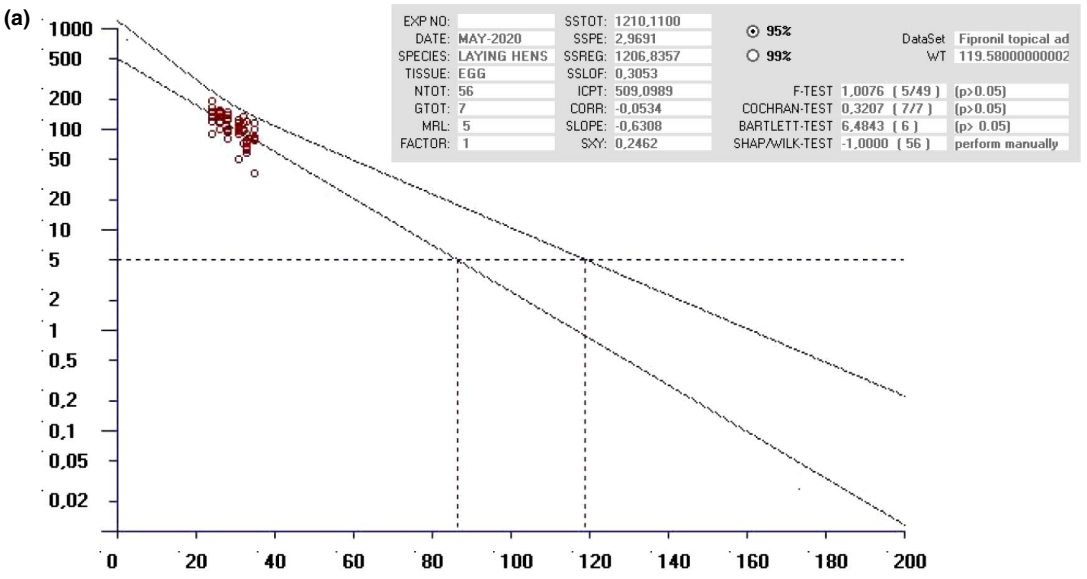
As mentioned above, the lack of drugs to treat *D. gallinae* has led to the illegal use of substances, such as fipronil. This was made evident by the fact that more than one in seven egg and chicken samples submitted by member states to EFSA contained fipronil residues exceeding legal limits (Reich & Triacchini, 2018). In Argentina, fipronil is authorized to treat ectoparasites in cattle and it is available as a 'pour-on' formulation (Ectoline®, 1%). Therefore, this medication has been a tempting extra-label treatment option for poultry mites. From conversations with workers in the industrial poultry sector, we learned that the fipronil formulation is mainly administered orally in feed (Personal communication). Additionally, topical administration on the back of the hen is also used, mainly for domestic production. In the current work, we investigated the presence of fipronil/fipronil-SO_2 residues in eggs after the fipronil extra-label administration to laying hens and suggested a withdrawal period in eggs.

Fipronil has been reported to be widely metabolized in the liver by cytochrome P450 (Roques et al., 2012) when administered to animals, giving fipronil-SO_2 as the main metabolite in many species, such as mice (Hainzl & Casida, 1996), rats (Cravedi et al., 2013), poultry (Stewart, 1994) and humans (Mohamed et al., 2004). Fipronil can also undergo photolysis to a desulfinyl product (fipronil-desulfinyl) (Bobe et al., 1998; Bobe et al., 1998; Ramesh & Balsubramanian, 1999; Tomlin, 2000). The long half-life (150–245 hr) of fipronil in blood may reveal a slow release of fipronil or its metabolites from fat (Gupta & Milatovic, 2014). In addition, fipronil-SO_2 was shown to persist much longer in blood and tissues than fipronil (Cravedi et al., 2013; Leghait et al., 2009, 2010; Roques et al., 2012).

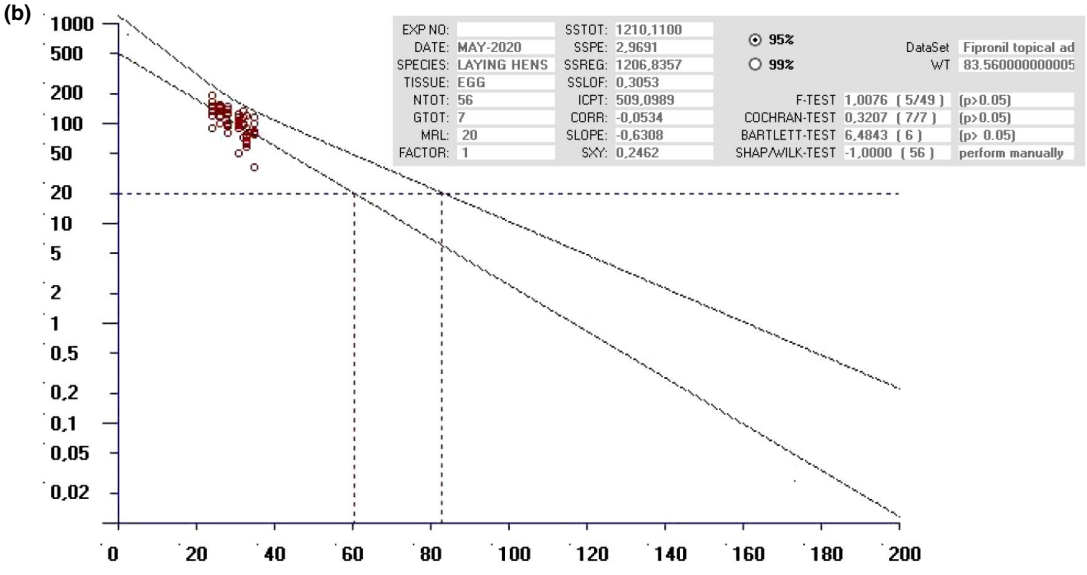
After fipronil administration to laying hens in feed, the drug was absorbed and distributed, reaching high residue levels of fipronil in egg. Fipronil parent drug was widely metabolized, giving the highest fipronil-SO_2 concentrations found in egg. Consequently, the permanence of fipronil residues was shorter (23 days) than that of fipronil-SO_2, which was quantified at very high concentrations until the end of the sampling period (35 days). This is a relevant finding as fipronil-induced toxicity has been fully associated with fipronil-SO_2 metabolite instead of the parent drug (Das et al., 2006; Romero et al., 2016).

After its topical administration, firponil was also absorbed and metabolized. However, its persistence in egg was shorter (16 days) and at lower residual concentrations than those observed after

(a)


EXP NO:
DATE: MAY-2020
SPECIES: LAYING HENS
TISSUE: EGG
NTOT: 56
GTOT: 7
MRL: 5
FACTOR: 1
SSTOT: 1210,1100
SSPE: 2,9691
SSREG: 1206,8357
SSLOF: 0,3053
ICPT: 509,0989
CORR: -0,0534
SLOPE: -0,6308
SXY: 0,2462
95%
99%
DataSet Fipronil topical ad
WT 119.58000000002
F-TEST 1,0076 (5/49) (p>0.05)
COCHRAN-TEST 0,3207 (7/7) (p>0.05)
BARTLETT-TEST 6,4843 (6) (p> 0.05)
SHAPWILK-TEST -1,0000 (56) perform manually
1000
500
200
100
50
20
10
5
2
1
0,5
0,2
0,1
0,05
0,02
0
20
40
60
80
100
120
140
160
180
200

(b)


EXP NO:
DATE: MAY-2020
SPECIES: LAYING HENS
TISSUE: EGG
NTOT: 56
GTOT: 7
MRL: 20
FACTOR: 1
SSTOT: 1210,1100
SSPE: 2,9691
SSREG: 1206,8357
SSLOF: 0,3053
ICPT: 509,0989
CORR: -0,0534
SLOPE: -0,6308
SXY: 0,2462
95%
99%
DataSet Fipronil topical ad
WT 83.560000000005
F-TEST 1,0076 (5/49) (p>0.05)
COCHRAN-TEST 0,3207 (7/7) (p>0.05)
BARTLETT-TEST 6,4843 (6) (p> 0.05)
SHAPWILK-TEST -1,0000 (56) perform manually
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(c)

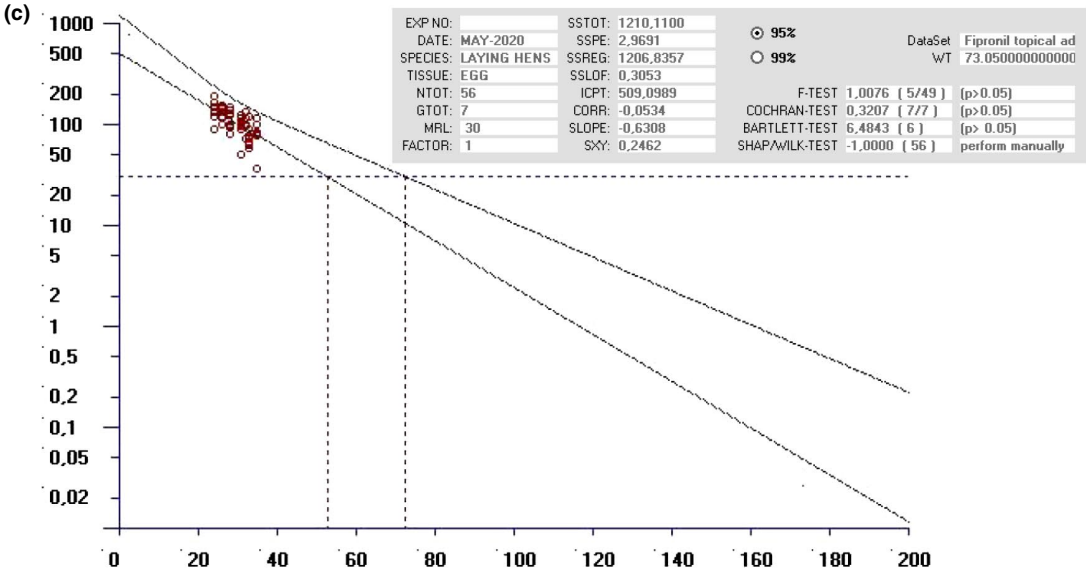
EXP NO:
DATE: MAY-2020
SPECIES: LAYING HENS
TISSUE: EGG
NTOT: 56
GTOT: 7
MRL: 30
FACTOR: 1
SSTOT: 1210,1100
SSPE: 2,9691
SSREG: 1206,8357
SSLOF: 0,3053
ICPT: 509,0989
CORR: -0,0534
SLOPE: -0,6308
SXY: 0,2462
95%
99%
DataSet Fipronil topical ad
WT 73.050000000000
F-TEST 1,0076 (5/49) (p>0.05)
COCHRAN-TEST 0,3207 (7/7) (p>0.05)
BARTLETT-TEST 6,4843 (6) (p> 0.05)
SHAPWILK-TEST -1,0000 (56) perform manually
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FIGURE 6 Withdrawal period calculations using the results of a linear regression analysis of the log residual concentrations *vs*. time of the terminal elimination phase after fipronil topical (a single dose of 1 mg kg^{-1} at day 1) administration to laying hens.. The 'Melk WTM 1.4' withdrawal-time calculation computer software (http://www.ema.europe.eu) was used. Different MRL or tolerance values were considered: 5 ng/g (Commission Regulation EU, 2019) (a); 20 ng/g (FAO/WHO, 2019) (b); 30 ng/g (tolerance) (Code of Federal Regulations, 2000) (c)

treatment in feed (fipronil ratio $C_{max\ oral\ in\ feed}/C_{max\ topical}$ = 8.43). Fipronil-SO_2 was also the main residue observed until the end of sampling, though at a much lower level than that observed after fipronil-medicated feed administration (fipronil-SO_2 $C_{max\ oral\ in\ feed}/C_{max\ topical}$ ratio = 11.3). These results were expected as two doses (1 mg/kg) were administered orally, while a single dose (1 mg/kg) was given by the topical route. However, if we consider partial AUC from 0 to 7 days ($AUC_{0\text{-}7\ days}$), it represents fipronil egg residue disposition after a single administration by either the oral or the topical route. When the fipronil partial $AUC_{0\text{-}7\ days}$ was compared between routes, statistical differences were found (p = 0.0005). The comparison of fipronil-SO_2 $AUC_{0\text{-}7\ days}$ values also showed statistical differences (p < 0.0001). Therefore, fipronil residues after topical administration were significantly lower than those obtained after oral administration in feed.

There is little information available on the fipronil/metabolites kinetics and egg residues after fipronil administration to laying hens by any route. To our knowledge, only three studies reported fipronil/metabolite residues in eggs, but fipronil was administered for longer periods than that in the current work. When fipronil was administered daily at 0.05, 2 or 10 ppm by the oral route (prior to feeding) for 28 days (Stewart, 1994), the mean maximum fipronil marker residues in egg yolk were 180; 7,020 and 30,000 ng/g, one day after the end of treatment, respectively. In keeping with the present work, the main residue was fipronil-SO_2 and it was described as a plateau at the end of the study (Stewart, 1994). Although we administered a much lower fipronil dose, only two 20 ppm doses in feed one week apart, the mean egg marker residue level was also considerable, with a C_{max} (1,863 ± 866 ng/g) attained at day 22, 15 days after the end of treatment. In another trial, laying hens were administered low fipronil doses of 0.01 ppm, 0.031 ppm or 0.103 ppm daily in their diet for 42 days (Byrd, 1994). Again, fipronil egg residues reached a plateau at about 25–28 days. Although fipronil-SO_2 was also the main analyte quantified in eggs, the mean levels at the end of the study were lower (10, 24 and 96 ng/g) (Byrd, 1994) than those found in the present and in the previous work (Stewart, 1994). More recently, we were able to collect samples from a laying hen farm, which was apparently administering extra-label fipronil (Ectoline®, 1%). Fipronil-SO_2 residual concentrations were quantified in yolk with a maximum residue level (C_{max}) of 2,100 ± 340 ng/g 9 days (t_{max}) after the beginning of treatment (Canton et al., 2018). Although total egg samples (not only yolk) were analysed in the present work, those residues were in the same range. Anyway, all the reported fipronil administrations exceeded the allowed tolerance/MRLs (Code of Federal Regulations, 2000; FAO/WHO, 2019; Commission Regulation EU, 2019). McCorquodale et al. (1996) gave oral capsules of fipronil-desulfinyl at 0.05, 2 and 10 ppm doses to laying hens for 14 days. In this case, fipronil-desulfinyl was the only residue in egg white and yolk, also reaching a plateau. One day after the end of the treatments, maximum egg residues for each dose level were 58, 1550 and 8700 ng/g (McCorquodale et al., 1996).

The long persistence of fipronil residues in egg is explained by its high lipophilicity, which determines its accumulation in this tissue with high lipid content. In some pet formulations, such as Frontline®, fipronil lipophilicity allows drug accumulation in fat and sebaceous glands, from which it is slowly released, providing a long action period of about 30 days (Frontline technical report, 2013). However, the scenario is different for food-producing animals, in which this characteristic translates into long withdrawal periods. The fat-soluble drug residue kinetics in eggs is peculiar compared to that observed in other edible tissues, in which an equilibrium tissue blood is established. Taking into account, the physiology of egg formation allowed us to understand why high fipronil residues were found in the egg. Like other described drugs (Donoghue et al., 1997; Marmulak et al., 2015; Moreno et al., 2018), fipronil administered to laying hens by both routes is absorbed and it reaches the ovary, follicles and oviduct. The follicles go through three phases until they become eggs. Phase 1 follicles/white follicles are immature without carotenoids (Kan & Petz, 2000). The arrival of fipronil residues would be significant for follicles that are in phase two/intermediate development, between 6 and 2 weeks before the egg is laid. The formation of the yolk begins with the arrival through the blood of lipoproteins from the liver, which accumulate in the yolk. Similarly, due to its fat solubility, fipronil would also arrive from the liver and fatty tissues and begin to accumulate within the developing follicle. However, the main fipronil accumulation must occur in the final stage of egg development (phase 3), between 14 and 10 days before the egg is laid because there is a rapid accumulation of yolk lipoproteins. This explains why we found high fipronil/metabolite residues in eggs for so long even though the fipronil administration was short. For example, the follicles that were in stage 2 at the time of the fipronil administration received residues directly after absorption, but fipronil accumulation continued due to its redistribution from the fatty tissues until the egg was laid approximately 6 weeks later (42 days). This is the reason why residues easily exceed the allowed limits after fipronil administration to laying hens (Canton et al., 2018; Reich & Triacchini, 2018). Certainly, in the current work, fipronil marker residues after both oral and topical administrations were above the mentioned tolerance/MRLs (Code of Federal Regulations, 2000; FAO/WHO, 2019; Commission Regulation EU, 2019). To our knowledge, we estimated for the first time the withdrawal period after fipronil administration to laying hens. The residue profiles found after both administrations and MRLs were used for calculations, and long withdrawal period was obtained. The shortest withdrawal periods after the topical administration were found to be 74, 84 and 120 days. These withdrawal periods are close to those indicated for

cattle meat after fipronil 'pour-on' administration (Ectoline®), which covers 100 days. However, from a practical point of view, fipronil is not suitable for use in egg production.

To conclude, the results reported here provide useful information on fipronil extra-label treatments to laying hens. Both oral and topical administrations led to long time residues in egg. Egg residual levels after fipronil administration in feed were significantly higher in comparison with the topical treatment. The long withdrawal periods estimated after both administrations hinder the use of fipronil in egg production. Therefore, its administration to laying hens should not be recommended under any circumstances. It is important to conduct educational campaigns aimed at poultry farmers to avoid fipronil extra-label use. Furthermore, it is crucial that the authorities responsible for the control of drug and residues in food (applicable to many other countries worldwide) implement a strict control of fipronil residues in eggs to guarantee the quality of this important food source, and therefore, the health of the consumer.

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CONFLICT OF INTEREST

There is no potential conflict of interests associated with this study.

AUTHOR CONTRIBUTIONS

L. Cantón has contributed to the experimental development of the work, preparing the medicated food, administering the medication and controlling the sampling. She has also been in charge of the analysis of results and writing of the manuscript. She has read and approved the final manuscript. C. Cantón has contributed to the validation of the analysis methods, the preparation, extraction and analysis of the samples. She has read and approved the final manuscript. L. Ceballos has contributed to the validation of the analysis methods, preparing and extracting samples. She has read and approved the final manuscript. P. Domínguez has contributed to the animal health control and selection of the animals, helping in the administration of the medication and taking/preparing samples. She has read and approved the final manuscript. J. Rodríguez has contributed to the care of the animals and taking samples. He has read and approved the final manuscript. C. Lanusse has participated in the correction of the manuscript. He has read and approved the final manuscript. L. Álvarez has contributed to the work design, the treatment administration and correction of the manuscript. He has read and approved the final manuscript. L. Moreno has contributed to the design, organization and experimental development of the work. She has also been in charge of the analysis of results and writing of the manuscript. She has read and approved the final manuscript.

ANIMAL WELFARE AND ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have adhered to international standards for the protection of animals used for scientific purposes.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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