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DETERMINATION OF NICARBAZIN AS DINITROCARBANILIDE RESIDUES IN CHICKEN FEED, BREAST AND LITTER

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ABSTRACT – To control coccidiosis, a common disease in commercial broiler production, anticoccidials are added to feed. However, concerns about the deposition of anticoccidial residues in chicken breast do exist. Brazilian law allows $200 \mu\text{g kg}^{-1}$ of nicarbazin (main chicken anticoccidial) residue in chicken breast, but demands its withdrawal from feed 10 days before slaughter, to avoid its presence in chicken breast. The present research aimed at raising chickens for 42 days and subject them to three treatments with anticoccidials by analyzing nicarbazin residues as dinitrocarbanilide (DNC) in feed, breast and poultry litter. The results showed that feed and breast had DNC levels within the legislation, therefore chicken breast is safe for human consumption. Also, shortly after nicarbazin removal from feed, DNC concentration dropped in poultry litter by about 50% in all treatments.

RESUMO – Para controlar a coccidiose, doença comum na produção comercial de frangos de corte, são adicionados anticoccidianos à ração. No entanto, preocupações sobre a deposição de resíduos de anticoccidianos no peito de frango existem. A legislação brasileira permite $200 \mu\text{g kg}^{-1}$ de resíduo de nicarbazina (principal anticoccidiano) no peito de frango, porém exige que seja retirado da ração 10 dias antes do abate, para evitar sua presença no peito de frango. A presente pesquisa objetivou criar frangos por 42 dias e submetê-los a três tratamentos com anticoccidianos, analisando resíduos de nicarbazina na forma de dinitrocarbanilida (DNC) na ração, peito e cama de aviário. Os resultados mostraram que na ração e no peito, todos níveis de DNC se mantiveram dentro da legislação, sendo o peito de frango seguro para consumo humano. Ainda, logo após a retirada da nicarbazina da ração, a concentração de DNC na cama de aviário caiu cerca de 50% em todos tratamentos.

KEYWORDS: chicken litter; veterinary drugs; regulation; broiler.

PALAVRAS-CHAVE: cama de aviário; medicamentos veterinários; legislação; frangos de corte.

1. INTRODUCTION

With the notable increase of worldwide consumption of veterinary medicines, the presence of drug residues in foodstuffs of animal origin is inevitable. Application of a higher dose than recommended, failure to respect proper withdrawal times, or irresponsible use can result in unwanted occurrence of drug residues in food of animal origin (Rocca et al., 2017).

Veterinary medicines or feed additives in food production are intended to control and improve animal health (Chapman, 2014). One common disease in poultry intensive production is coccidiosis, caused by protozoals (spp. *Eimeria*) (Clarke et al., 2014), responsible for economic loss; therefore much attention has been given to

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this disease which is prevented by anticoccidial use. Consequently, many coccidiostats are licensed as feed additives and administered in feed (Chapman, 2014; Clarke et al., 2014). Nicarbazin (NCZ) is among the most widely used anticoccidials to control coccidiosis in poultry production chain. It consists of an equimolar complex of 2-hydroxy-4,6-di-methylpyrimidine (HDP) and 4,4'-dinitrocarbanilide (DNC). While the former is excreted rapidly after the additive withdrawal from feed, the latter is slowly eliminated and is thus considered of concern in chicken meat (EFSA, 2010). Coccidiostats are potent drugs and may exacerbate certain coronary disease conditions when found in food. It is important, therefore, for poultry, milk, meat and egg producers to prevent the occurrence of these residues in food products (Rocca et al., 2017).

The Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA) defined the maximum concentration of NCZ allowed in feed (125 mg kg^{-1}) and recommended 10 days as its withdrawal period from the feed. Regarding the maximum residue limit (MRL), Brazil follows *Codex Alimentarius* allows up to $200 \text{ } \mu\text{g kg}^{-1}$ NCZ as DNC in raw chicken meat (BRASIL, 2019). The USA and Canada are more permissive by allowing $4000 \text{ } \mu\text{g kg}^{-1}$ NCZ in chicken meat (Delatour et al., 2018).

As Brazil produced almost 13 million tons of chicken meat in 2018, besides led exports in about 4 million tons (ABPA, 2019), the country has to provide scientific reliable data to the society and to regulatory agencies in order to negotiate exports when a barrier is imposed by a foreigner country. Lately, many countries are demanding prove that Brazil is able to provide safe meat and although it can, most of the times, is lacking scientific background to support official decisions. Hence, adequate analytical methodologies are necessary for an effective control of the absence of such residues and for safety assurance (Toldrá & Reig, 2014). LC-MS is a versatile technology, broadly accepted and recognized for both quantification and screening of chemical contaminants in food, and specifically veterinary drug residues (Delatour et al., 2018). Therefore, this work aimed at determining NCZ as DNC in feed, chicken breast and litter, by raising chickens during 42 days.

2. MATERIAL AND METHODS

Field experiment and sampling. Sixteen male and sixteen female chickens were raised in each box, according to the Treatments detailed in Table 1. The experiment was done in *Embrapa Suínos e Aves*. Feed samples were collected in the beginning of each experimental phase (starter = day 1, growing = day 22, finishing = day 33). Litter samples were collected at days 1, 21, 35 and 42 by mixing 5 different collecting points inside the box in order to compose a unique sample per box. NCZ given at 125 mg kg^{-1} in animal feed is equal to 89 mg kg^{-1} of DNC (considering an equimolar ratio with HDP). Maduramicin (MAD) premix consists of 0.75% MAD + 8% NCZ and its inclusion in animal feed is 500 g ton^{-1} . Monensin (MON) is the negative control.

Table 1 – Treatments according to chicken phase of life and different administered anticoccidials

Treatment	Starter (1-21 days)	Growing (22-32 days)	Finishing (33-42 days)
T1	NCZ (125 mg kg^{-1})	no NCZ, with MON	no NCZ, with MON
T2	NCZ (125 mg kg^{-1})	NCZ (125 mg kg^{-1})	no NCZ, with MON
T3	NCZ (40 mg kg^{-1}) + MAD	NCZ (40 mg kg^{-1}) + MAD	no NCZ, with MON

NCZ = nicarbazin; MAD = maduramicin premix; MON = monensin.

Extraction of NCZ from feed samples. The method used was adapted from Protasiuk et al. (2015). Feed samples from chickens fed no NCZ were obtained to implement the method. Feeds containing 40 and 125 mg kg^{-1} of NCZ ($n = 4$ for each concentration) were produced in *Embrapa Suínos e Aves*. About 2 g of homogenized samples were weighed in 50 mL Falcon tubes. Then, 20 mL of 90:10 acetonitrile/water (v/v) were added and the mixture was stirred by inversion for 30 min. The mixture was centrifuged at $4000g$ ($15 \text{ }^\circ\text{C}$; 10 min). Solid phase extraction cartridges containing C18 (500 mg) were previously conditioned with 90:10 acetonitrile/water ($2 \times 2 \text{ mL}$). Then, 2 mL of the supernatant from the centrifuged solution was applied to the cartridges, which were eluted by gravity with 90:10 acetonitrile/water ($2 \times 2 \text{ mL}$). The eluates from each sample were combined in a single 15-mL tube and the resulting solution was evaporated under a flow of N_2 at $45 \text{ }^\circ\text{C}$ to 2 mL. The sample solutions



were transferred to 1.5-mL vials and analyzed by HPLC-UV. Quantification was based on a calibration curve prepared in the matrix (NCZ-free feed) with DNC concentrations between 1 and 20 mg kg⁻¹.

Extraction of NCZ from chicken litter. About 2 g of the previously dried samples were weighed in 50 mL Falcon tubes. Then, 20 mL of acetonitrile (ACN) was added. The mixture was subjected to ultrasound for 5 min and then stirred by inversion for 30 min. The mixture was centrifuged at 4000g (15 °C; 10 min). Then, 800 µL of the supernatant were transferred to a 15 mL tube containing 180–200 mg of C18 adsorbent. Ultrapure water (200 µL) was added and vortexed vigorously for 1 min. The solution was filtered through a 0.45 µm (25 mm) membrane and the filtrate poured into a 200 µL insert stored within 2-mL vials. The sample solutions were quantified by HPLC-UV based on a calibration curve prepared in the matrix (NCZ-free litter) with DNC concentrations between 1 and 20 mg kg⁻¹.

HPLC-UV analysis of NCZ. Solutions resulting from NCZ extraction from feed and litter samples were injected into a Thermo-Scientific™ Dionex™ Ultimate 3000 HPLC system equipped with a diode array detector (DAD). An Acclaim™ 120 C18 analytical column (150 mm x 4.6 mm, 5 µm) was used for chromatographic separations. Column temperature: 30 °C. Autosampler compartment temperature: 10 °C. Injection volume: 10 µL. The mobile phase was composed by 60% ACN with 40% of 200 mmol L⁻¹ ammonium acetate solution. The elution was performed in isocratic mode at a flow rate of 0.6 mL min⁻¹. DAD has been programmed to monitor UV absorbance at 364 nm.

Extraction of DNC from chicken breast. First, about 1.5 g (equivalent to 5 g in wet basis) of ground freeze-dried sample was weighed into a 50 mL Falcon tube and fortified with 200 µL deuterated DNC (DNC-d₈) internal standard (IS). Subsequently, sodium sulfate (1.0 g) was added, and stirring by vortex for 15 s. Acetonitrile (20 mL) was added and the tube was shaken in a vortex for 30 s. Then, the tube was shaken for 30 min on a “wrist action” shaker. Centrifugation was carried out at 3500g and 5 °C for 10 min. The supernatant was transferred to another 50 mL conical tube, the sample pellet was re-extracted with ACN (20 mL), and supernatants were combined and the volume adjusted to 50 mL with ACN. NCZ working solutions were added to blank sample extracts to daily prepare a matrix-matched analytical curve with an IS. Sample extract was filtered (Millipore Millex-GV filter unit, 0.22 µm) into LC vial and stored at -20 °C until DNC determination by LC-MS/MS.

LC-MS/MS analysis of DNC. The analyses were performed in a LC System Surveyor Plus (Thermo, USA). Separations were carried out in a Kinetex C18 100 Å analytical column (100 × 4.6 mm, 5 µm pore size) combined with a C18 guard column. Column temperature was 30 °C. Injection volume was 10 µL. A combination of two mobile phases (A and B) was used at a flow rate of 1.0 mL min⁻¹. Mobile phase A consisted of methanol and 0.1% formic acid (v/v). Mobile phase B consisted of water and 0.1% formic acid (v/v). Separations were achieved with a gradient program described as follows: 5% A (0–1 min), 80% A (1–2 min), 100% A (2–5 min, maintained until 7 min), 5% A (7–7.5 min and held until 11 min). The autosampler was operated at 10 °C.

MS measurements were carried out on a triple-quadrupole mass spectrometer Quantum Access Max (Thermo, USA). DNC and internal standard DNC-d₈ (10 mg L⁻¹ in ACN) solutions were directly infused (at 10 µL min⁻¹) for MS spectrometer tuning in negative electrospray ionization mode. The precursor ions and the respective product ions of DNC and DNC-d₈ were identified. In addition, the spray voltage (3.5 kV) and the collision energies (CE) were optimized. For DNC, the deprotonated molecular ion [M-H]⁻ at *m/z* 301.0 was selected as the precursor ion, while the product ions at *m/z* 107.2 (CE 40 eV) and *m/z* 137.1 (CE 21 eV) were set for quantification and confirmation, respectively. The deprotonated molecular ion [M-H]⁻ at *m/z* 308.7 was selected as the precursor ion for DNC-d₈ whose ion product at *m/z* 141.2 (CE 19 eV) was set for quantification. Retention time was also used for analyte confirmation. With infusion of the same solution of the molecules into the MS spectrometer with mobile phase (50:50 water/methanol, both with 0.1% formic acid) at 1 mL min⁻¹, the source conditions were optimized, as follows: vaporized temperature at 261 °C; capillary temperature at 360 °C; sheath gas pressure at 50 psi; auxiliary gas pressure at 5 psi. Nitrogen was used as nebulizer gas and argon as collision gas at a pressure of 1.8 mTorr.

3. RESULTS AND DISCUSSION

The control treatment diet had 0 mg kg⁻¹ in all treatments. As can be seen (Table 2), the birds received no nicarbazin (NCZ) in feed in the last 10 days obeying the Brazilian legislation, thereby no residues were detected in feed and amounts below the MRL were detected in their breast (Figure 1). Although along chicken life, at each feed change phase (starter to growth; growth to finishing), NCZ and DNC were detected, with the exception of T1 growth (Table 2). However, if the concentrations added to feed are respected (up to 125 mg kg⁻¹), no residues should occur in chicken breast.

Table 2 – Nicarbazin in chicken feed according to three treatments and phases of life

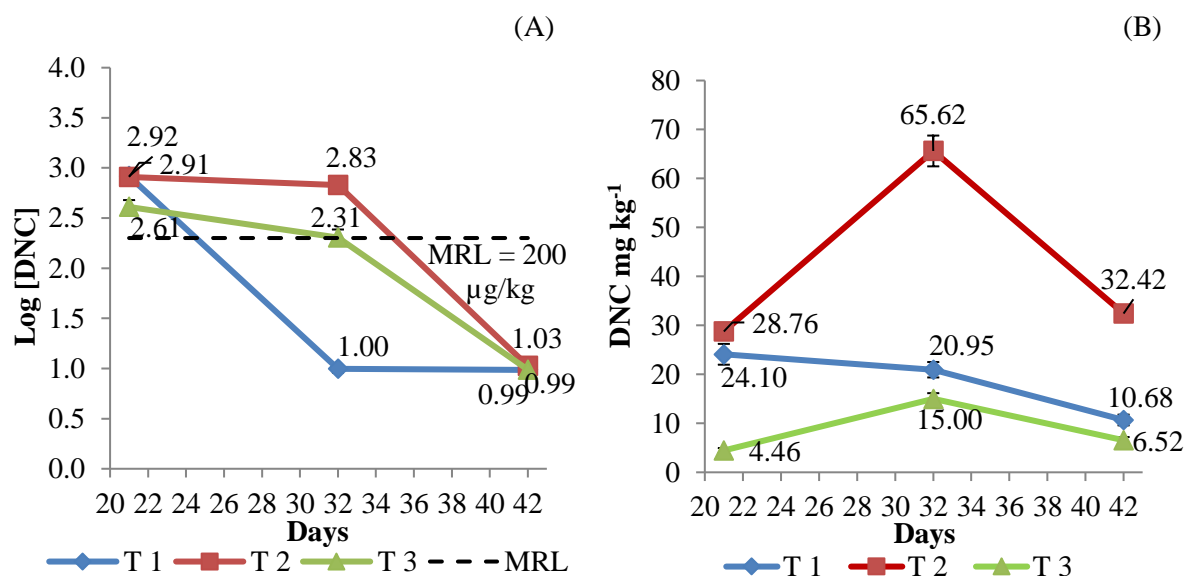
Phase	Age (days)	T	DNC formulated (mg kg ⁻¹)	NCZ formulated (mg kg ⁻¹)	DNC found (mg kg ⁻¹)	NCZ found (mg kg ⁻¹)
Starter	1 a 21	1	89	125	77	109
Starter	1 a 21	2	89	125	77	109
Starter	1 a 21	3	28	40	30	42
Growth	22 a 32	1	0	0	0	0
Growth	22 a 32	2	125	106	89	75
Growth	22 a 32	3	40	39	28	28
Finishing	33 a 42	1, 2, 3	0	0	0	0

T = treatment

According to Figure 1A, although DNC concentrations are high in the beginning of the treatments (T1, T2, T3), which is expected, once the anticoccidial is exerting its effect to protect the animals against common disease during rearing, the concentration was below the MRL at the time of slaughter (42 days), therefore, the amounts encountered herein present no risk to consumers that eventually eat this chicken breast. In T1, as no NCZ was added at growing (22 days), when the birds reached 32 days of age or even before, animal breast was already clean from DNC residues. Similar behavior was observed among T2 and T3, while the former was above (2.83) the MRL at 32 days, the other was around (2.31) the MRL. Considering the withdrawal time of 10 days before slaughter similar to the growing phase period of about 10 days (day 22 – 32), this period can be recommended as safe in case an urgent or anticipated slaughter is needed, but only when no NCZ is given at growing phase (T1).

Lima et al. (2017) evaluated different withdrawal times when feeding broiler with NCZ and narasin. They concluded that 4 days of NCZ withdrawal was enough to ensure no DNC residues in chicken breast already at 32 days of age. However, when NCZ is administered alone, these researchers recommended 6 days of withdrawal time from feed.

Figure 1 – Dinitrocarbanilide (DNC) residues, expressed as means \pm standard error, in breast (A) and litter (B) of chickens submitted to three treatments with anticoccidials.



T1 (Figure 1B) showed different behavior from the other treatments, because no NCZ was given from the growing phase on. Therefore, decrease in DNC concentration was observed since 21 days of animal age. Otherwise, T2 and T3 had an initial increase up to 32 days, then decreased soon after NCZ was withdrawn from the feed. Also, after NCZ withdrawal at 32 days in all treatments, DNC residue concentration naturally decreased nearly 50%.

Litter undergoes treatment by covering its extent completely to allow fermentation for 10-12 days before it can be reused (Silva, 2011). So, probably DNC concentration will decrease even more, what implies in litter reuse for the next lot, which is a benefit to the farmers and agroindustries that raise chicken for profitability. A survey from Israel (MacDonald et al., 2000) that also used 125 mg kg⁻¹ of NCZ in feed showed DNC in litter varying from 17 to 68 mg kg⁻¹, values similar to the present research. These scientists stated that DNC aqueous solubility is low (0.02 $\mu\text{g mL}^{-1}$) and mixed with feces leads to limiting intestinal absorption by chickens in case they pick litter and ingest it.

In Brazil, a pioneer study was conducted by Penz et al. (1999), that evaluated DNC concentration in tissues of broilers in three consecutive lots, with reuse of poultry litter from one lot to another, considering the additive withdrawal period of 7 days before slaughter. Nicarbazin was added in different concentrations (100 and 125 mg kg⁻¹ in feed) as the only anticoccidial and combined with maduramicin (40 mg kg⁻¹ added in feed). Regarding the tissues studied, the liver showed the highest concentration of residues, regardless of the lot. In addition, it was observed that the residues persisted in the litter for more than two lots after the removal of nicarbazin from the feed.

Our upcoming experiments will evaluate up to ten lots reusing the same litter. We encourage more research in this area, because the literature is scarce and the population need to be aware if they are consuming healthy products. In the same way, constant training to the producers in the field should be given in order to them complying with the legislation and assure the proper use, so the possibility of residues in tissues will be minimized.



4. CONCLUSION

The dinitrocarbanilide (DNC) residues found in chicken breast were far below the MRL set by worldwide legislation at the time of slaughter. Feed showed DNC profile was within expected, obeying legislation. Also, DNC residues in chicken litter fell around 50% comparing growing with finishing phase. Further studies with longer lots are needed to address how long can chicken litter be reused to ensure safety.

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