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**PHARMACOKINETICS STUDY OF CLAZURIL  
(APPERTEX®) IN EGGS AND PLASMA FROM  
LAYING HENS AFTER SINGLE AND MULTIPLE  
TREATMENT BY A NEW HPLC METHOD.**

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► **To cite this version:**

Mario Giorgi, Giulio Soldani. PHARMACOKINETICS STUDY OF CLAZURIL (APPERTEX®) IN EGGS AND PLASMA FROM LAYING HENS AFTER SINGLE AND MULTIPLE TREATMENT BY A NEW HPLC METHOD.. British Poultry Science, 2008, 49 (05), pp.609-618. 10.1080/00071660802294566 . hal-00545339

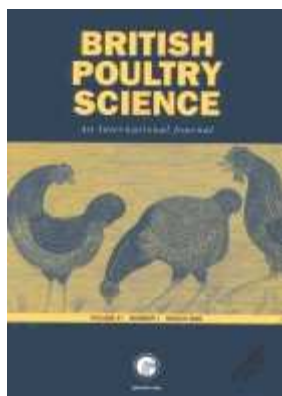
**HAL Id: hal-00545339**

**<https://hal.science/hal-00545339>**

Submitted on 10 Dec 2010

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**PHARMACOKINETIC STUDY OF CLAZURIL (APPERTEX®) IN EGGS AND PLASMA FROM LAYING HENS AFTER SINGLE AND MULTIPLE TREATMENT BY A NEW HPLC METHOD.**

Journal:	<i>British Poultry Science</i>
Manuscript ID:	CBPS-2007-298.R2
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	01-Jun-2008
Complete List of Authors:	Giorgi, Mario; University of Pisa, Section of Pharmacology & Toxicology Department of Veterinary Clinics Soldani, Giulio; Section of Pharmacology & Toxicology, Veterinary clinics
Keywords:	Pharmacology, Laying hens, Egg



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3 **Pharmacokinetic study of Clazuril (Appertex<sup>®</sup>) in eggs and plasma from laying hens after**  
4 **single or multiple treatments, using a new HPLC method for detection**  
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32 Accepted for publication 11 June 2008  
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37

38 **Abstract.** 1. Anticoccidials are widely used as food additives to prevent and treat coccidiosis. They  
39 are licensed for use in a prescribed concentration and during a specific time interval with broilers  
40 and pullets, but not for laying hens.  
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45  
46 2. This study was set up to develop a new high pressure liquid chromatography (HPLC) method to  
47 detect clazuril (CZ: ( $\pm$ )-2-chloro- $\alpha$ -(4-chlorophenyl)-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin)-2(3H)-  
48 yl)-benzeneacetonitrile) in egg yolk and albumen and in plasma; to investigate both the presence of  
49 residues of CZ in eggs and its pharmacokinetic behaviour in laying hens.  
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54  
55 3. A single oral dose (3 mg/kg BW) and multiple oral doses (3 mg/kg BW for 5 days) were  
56 investigated. The analytical method gave very good recovery (64-74%) in the three different  
57 matrices (yolk, albumen and plasma); precision and accuracy were within 11%.  
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3 4. After a single dose no residue was detected in eggs collected for up to 10 d, while following  
4  
5 multiple dose treatment, CZ residues were detected until 10 d after the end of treatment. The  
6  
7 concentration of the drug was higher in yolk than in albumen with a maximum ratio of 10:1.  
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10 5. Pharmacokinetics of CZ in laying hens after a single dose showed a detectable concentration of  
11  
12 the drug up to 24 h. It reached a steady state after the third administration in multiple dosing.  
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15 6. Although further studies are necessary, these results indicate that a single oral dose of CZ could  
16  
17 be used as an anticoccidial for laying hens due to the lack of residues in eggs.  
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## INTRODUCTION

Anticoccidials (AC) are widely-used for preventing and treating coccidiosis; an infection affecting livestock, especially poultry, throughout the world, and causing intestinal lesions, which result in diarrhoea and related health problems in many animals (Mortier *et al.*, 2005). The disease is carried by protozoa belonging to the genus *Eimeria* (class Sporozoa). In its acute form coccidiosis in poultry results in high mortality; in its subacute form, small numbers of oocysts are produced with adverse effects on weight gain, food conversion and egg production. Of all domestic species, commercially-bred poultry and rabbits are particularly prone to this disease. The economic damage caused by coccidiosis in modern poultry production is so serious that practically all poultry farms have resorted to using either vaccines or AC as feed additives for pullets and broiler breeders for up to 12 - 16 weeks and to broiler chicks for almost their entire lives. Despite the use of AC drugs, coccidiosis remains one of the greatest causes of financial loss in poultry production.

AC can be used at a prescribed concentration and for a specific time interval with broilers and pullets but not with laying hens. No residues of anticoccidials must be present in eggs. Increasingly strict regulations or bans on the use of anticoccidial drugs, coupled with the crippling cost of developing new drugs or live vaccines has increased the need to develop novel approaches or alternative control strategies for coccidiosis. The poultry industry has obtained excellent results with the first large-scale trials of a live-attenuated coccidial vaccines for use with broiler chickens. Currently several vaccines have been registered for use with chickens or turkeys in various countries (Chapman, 2000; [Dalloul and Lillehoj, 2006](#)). However, in many countries AC are still used and carry-over in the feed mill is a major problem, amounting to 15% contamination (Kennedy *et al.*, 1998), consequently AC can be present in feed intended for laying hens.

With a view to a decision on the phasing-out of anticoccidials as feed additives by December 31, 2012, the European Commission is required to submit to the European Parliament and Council a report on the use of such substances as feed additives, and the available alternatives, in 2008. At present, there are no maximum residue levels (MRL) set for eggs (EEC, 1990: Council

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3 Regulation 2377/1990). Hence the zero tolerance principle is applied and consequently AC must not  
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5 be present in eggs. In practice, however, different EU member states apply different approaches. In  
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7 Belgium, an action limit of 10  $\mu\text{g}/\text{kg}$  has been proposed by the Scientific Committee of the Belgian  
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9 Food Agency for: monensin, salinomycin, diclazuril, lasalocid, maduramycin, narasin, nicarbazin,  
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11 robenidine and the group of sulfonamides (www.favv.be.).  
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15 Toltrazuril (TZZ), diclazuril (DCZ) and clazuril (CZ) are three AC agents with a similar  
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17 molecular structure (Figure 1). TZZ is intended for use in poultry, pigs, beef cattle and all other  
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19 mammalian food-producing species for the prevention and treatment of coccidiosis, but its use is  
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21 not approved for animals from which milk and eggs for human consumption are produced. DCZ has  
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23 been registered as an anticoccidial feed additive for broilers with a withdrawal period of 5 d, and  
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25 subsequently approved, for oral use only, in porcine and all ruminant species. CZ, ( $\pm$ )-2-chloro- $\alpha$ -  
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27 (4-chlorophenyl)-4-(4,5-dihydro-3,5-dioxo-1,2,4-triazin)-2(3H)-yl)-benzeneacetonitrile, is an AC  
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29 that was initially intended for oral use in pigeons under the trade name Appertex<sup>®</sup>. The  
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31 recommended dosage is 2.5 mg CZ (one tablet) per pigeon per month. Although the mode of action  
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33 of CZ is not precisely known, its effect on the asexual or sexual stages of coccidia blocks the  
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35 excretion of oocysts, interrupting the life cycle of the parasite. Few papers have been published on  
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37 the efficacy of this drug in pigeons (Vanparijs *et al.*, 1988; Maes *et al.*, 1988; Coussement *et al.*,  
38  
39 1988; Vercruyse, 1990; Carpenter *et al.*, 2005). No analytical method for detection of CZ in any  
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41 medium has been reported. The drug is listed in Annex II (EEC, 1990: Council Regulation  
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43 2377/1990) and it appears that, for the protection of public health, it is not necessary to establish an  
44  
45 MRL, for substances included in Annex II; however, it should be noted that an entry in Annex II is  
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47 not equivalent to the status “generally recognised as safe”. No pharmacokinetic and radiometric  
48  
49 studies using laying hens have been done. The pharmacokinetic behaviour of CZ has been  
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51 extrapolated from studies, carried out on broiler chickens and turkeys, using the dichloro analogue  
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53 DCZ, relying on structural analogy between the two compounds. The aim this study was to develop  
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55 an analytical HPLC method to evaluate both the pharmacokinetics of CZ in laying hens and its  
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presence in the eggs (yolk and albumen) after single or multiple treatments with Appertex<sup>®</sup> using doses similar to those used for pigeons.

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## MATERIALS AND METHODS

### Apparatus

The liquid chromatography (LC) system consisted of a Thermo Finnigan Spectra system P2000 pump, with an Uv/Vis detector (Waltham, MA, USA) coupled by autosampler/sampleprocessor equipped with a variable 1-100  $\mu$ L loop. The LC column was a SunFire, 5  $\mu$ m, 4.6 x 250 mm, with guard column using the same packing (Waters, Milford, MA, USA). The LC mobile phases were filtered through 0.22  $\mu$ m nylon-66 (N-66) filters with a solvent filtration apparatus. Prior to injection on to the high pressure liquid chromatography (HPLC) apparatus, sample extracts were filtered through 13 mm 0.2  $\mu$ m Acrodisc nylon filters (Gelman, Ann Arbor, MI).

### Reagents

LC grade water was purified in-house with a Milli-Q Plus water system and was used for preparing all solutions. Acetonitrile (ACN), methanol (MeOH), and ethylacetate (EtOAc) were of super HPLC grade. CZ, DCZ and Toltrazuril (TTZ) were purchased from LGC Promochem (Bologna, Italy). The oral pharmaceutical formulation of CZ used for animal treatment was Appertex<sup>®</sup> (Janssen-Cilag, Milan, Italy). Solutions A and B consisted of 0.5% ammonium acetate + tetrabutyl ammonium hydrogen sulphate (0.01M) with CH<sub>3</sub>CN (80/20 v/v) and MeOH - CH<sub>3</sub>CN (80/20 v/v), respectively. All the other reagents and materials were of analytical grade and obtained from commercial sources.

### Standard solutions

#### *Stock solutions*

On the basis of the listed potency or purity of the standards, the amounts of CZ, DCZ and TTZ needed to prepare 100 mL of the individual standard solutions (1000  $\mu$ g/mL) was calculated. All AC concentrations are expressed as the free base equivalent. The standard was made up to the nearest 0.1 mg in a 100 ml volumetric flask and adjusted to 100 ml with water (for TTZ) or MeOH (for CZ and DCZ). These solutions were used immediately for the preparation of the fortification solution described below.



### *Fortification solution*

Twenty millilitres of each stock standard solution (1000  $\mu\text{g/ml}$ ) was pipetted into a 100 ml volumetric flask and adjusted to 100 ml with Milli-Q Plus water: this operation was repeated to obtain AC concentrations of 40  $\mu\text{g/ml}$ . These solutions were successively diluted in water to reach a final concentration of 5  $\mu\text{g AC/ml}$ . The final dilution was poured into a plastic tube (50 ml) and stored at  $-80\text{ }^{\circ}\text{C}$ .

### *Calibration standards*

The first dilution of fortification solution (200  $\mu\text{g AC /ml}$ ) was diluted with MeOH, to prepare a five-point standard curve for the AC at concentrations: 25, 50, 75, 100, and 150  $\mu\text{g/ml}$  (ppm). For concentrations at or below 20  $\mu\text{g/ml}$ , a standard curve of 0.1, 1, 5, 10, and 20  $\mu\text{g/ml}$  was prepared instead. These solutions were freshly prepared. Matrix-matched calibration standards were used to validate the method.

### **Animal experiments and sampling**

Animal experiments were conducted at the experimental poultry facility of Faculty of Veterinary Medicine (University of Pisa, Italy). Animal care and handling were performed according to the provisions of the EEC Directive 86/609 (EEC, 1986). The study protocol was approved by the local ethics committee of the University of Pisa (CASA).

Twenty four ISA-brown laying hens (weighing 1.7 - 2.2 kg) in the first half of their production cycle (31-39 weeks of age) were used. The hens were divided at random into two groups each of 12 animals and housed in single cages under conventional conditions of ventilation, temperature (18-22  $^{\circ}\text{C}$ ) and lighting (16 h light/d). During the study, they were given free access to water and food. Earlier, each group had been selected for laying consistency to improve the homogeneity of the entire flock. During the entire experiment, the hens were monitored daily for general health by qualified personnel supervised by a veterinarian. Eggs were collected daily during the complete course of the study. After the hens were placed in their pens, they were allowed to

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3 adjust to the new environment for 4 weeks. During this adjustment period, all were provided with  
4 AC-free food. Eggs collected during this period were used as the control material. After the  
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8 adjustment period, group I continued to receive drug-free food while the other group (group II) was  
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10 given a single dose of 3.0 mg CZ /kg BW in capsule form. After 10 days the group (II) was dosed  
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12 with 3 mg/kg BW of CZ in capsules daily on 5 consecutive days (multiple dose). Collection of the  
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15 eggs ended 21 d after cessation of drug administration. From each experimental group, about 10-12  
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18 eggs were collected daily; the yolk and albumen were separated and stored at -18 °C until analysis.  
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20 After a 14 d 'washout period', the treatments were repeated for groups 1 and 2 (using the same  
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22 animals), but in addition blood samples were collected (by brachial venipuncture) at 0, 1, 2, 3, 4, 5,  
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24 6, 7, 8, 9, 10 and 24 h, for the one day treatment group (single dose), with sampling every 7 and 24  
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26 h after each dosage for 10 d for the multiple-dosed group. Due to the limited volumes of blood  
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28 collected from each bird, blood samples from three laying hens were pooled before processing .  
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31 Blood samples were stored in tubes containing the anti-coagulant lithium heparin. Samples were  
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33 centrifuged within 30 min of collection and the plasma stored frozen at -20 °C until analysis.

### 36 Extraction procedure

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39 Egg yolk or albumen ( $2.0 \pm 0.1$  g) was weighed in disposable 15 ml polypropylene centrifuge tubes.  
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42 The fortification solution (5 µg/ml) was added at this stage and the mixture gently vortexed. Sodium  
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44 chloride (0.5 g) was added to each sample followed by 5 ml of organic solvent (MeOH or ACN),  
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46 and the sample was stirred using a spatula to ensure adequate mixing. The centrifuge tube was  
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48 capped and vortex-mixed at high speed for 30 s, followed by centrifugation for 5 min at 3000 g at  
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50 4°C to cause phase separation. The clear supernatant was transferred, using a disposable glass  
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52 pipette, into a separate 15 ml polypropylene centrifuge tube. Organic solvent (5 ml) was added, and  
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55 the extraction procedure was repeated one, two or three times to evaluate the influence of  
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60 number of extraction cycles. The supernatants were combined and centrifuged for 5 min at 3000

g at 4°C. The supernatant was transferred into a glass test tube and evaporated to dryness at 40°C under a N<sub>2</sub> atmosphere. Two millilitres of MeOH was added and the sample was vortexed. The contents were transferred into a disposable syringe fitted with a 13 mm Acrodisc nylon filter and filtered into a glass autosampler vial. One hundred microlitres were injected on to the LC system within 24 h of preparation. The extraction procedure for the plasma samples was the same as that used for eggs, starting with 1 ml plasma but adding 1 ml MeOH.

### Chromatographic conditions

Egg and plasma extracts were analysed for AC using the following isocratic LC conditions: mobile phase, consisted of CH<sub>3</sub>CN-H<sub>2</sub>O, CH<sub>3</sub>CN-NaH<sub>2</sub>PO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub> 0.1M, solution A-solution B, blended at different % and different pH (Table 1); flow rate, from 0.8 to 1.6 ml/min; wavelength, 222 and 244 nm (simultaneously detection); run time, from 7 to 15 min; column temperature, 25±0.5 °C. A CH<sub>3</sub>OH pure was injected to equilibrate the LC system. One hundred microlitres of each standard series was injected prior to injecting a sample set.

### Quantitation

A calibration curve of the peak area versus concentration (µg/ml) was plotted for each analyte. Least-squares regression parameters for the calibration curve were calculated, and the concentrations of the test samples were interpolated from the regression parameters. Sample concentrations were determined by linear regression, using the formula  $Y = mX + b$ , where  $Y$  = peak area and  $X$  = concentration of the standard in µg/ml (ppm). Correlation coefficients for each of the calibration curves were routinely >0.99. To estimate the concentration, the areas under the curve specific for CZ were calculated using the **internal standard** (IS) method according to equation:

$$CZ_{\text{area}} = (\text{plotted } CZ_{\text{area}} \times \text{DCZ recovery})/100.$$

When unknown samples are assayed, a control and a fortified control were processed along with each set for quality control.

## Pharmacokinetics and statistical analysis

Plots of plasma CZ concentration vs time were constructed for each pool of sera (n=4).

Pharmacokinetic analysis was performed, for each data set, using the computer program WinNolin version 3.1 (Pharsight Corporation, CA, USA). Akaike's information criterion (Yamaoka *et al.*, 1978) was used to select the best-fitting model (monoexponential vs biexponential). Parameters computed included AUC (h  $\mu\text{g/ml}$ ) area under the plasma concentration-time curve;  $K_{01}$  ( $\text{h}^{-1}$ ),  $K_{10}$  ( $\text{h}^{-1}$ ) microconstants that estimate the absorption of the drug in blood ( $K_{01}$ ), and its elimination from plasma ( $K_{10}$ ); half-life of the absorption phase ( $K_{01} t_{1/2}$ ), half-life of the elimination phase ( $K_{10} t_{1/2}$ ); CL ( $\text{ml}\cdot\text{h}^{-1}\cdot\text{kg}^{-1}$ ) total clearance; Tmax (h) times of peak plasma concentration; Cmax ( $\mu\text{g/ml}$ ) peak plasma concentration. One way analysis of variance (ANOVA) was applied to the principal pharmacokinetic parameters obtained from the different treatments and pools. Significance was assumed to be  $P < 0.05$ .

## RESULTS AND DISCUSSION

### Extraction solvent

Pure organic solvents, such as ACN or MeOH, were able to extract the target AC from the different egg and plasma matrices with different degrees of success; ACN was most effective solvent for extraction of CZ. MeOH caused the formation of an emulsion phase that was difficult to destroy even with prolonged centrifugation or by increasing the ionic concentration of the blend by adding NaCl. Therefore, ACN solvent was selected as the preferred solvent because it provided cleaner extracts and better precision. No significant differences were evident between albumen, yolk and plasma samples. In these three

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3 organic matrices TTZ showed lower recovery compared to DCZ and, consequently, was  
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7 excluded from the study.  
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### 10 **Number of extraction cycles**

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14 To investigate the effect of this variable on the efficiency of AC extraction from egg and  
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18 plasma matrices, a standard extraction time was set at 5 min and the number of extraction  
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22 cycles increased between one and three, with the corresponding increase in the time of  
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25 extraction from 5 to 15 min. No significant differences were found between the three  
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28 different organic matrices; to make the results easier to interpret, only those for yolk  
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31 samples have been shown (Figure 2). In general, an increase in the number of extraction  
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35 cycles allows exposure of the matrix to fresh solvent and favours the solvent/sample  
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38 equilibrium, improving partitioning into the liquid phase and thereby increasing analyte  
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41 recovery. In this particular case, the number of cycles did not increase the extraction  
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44 efficiency, because there were negligible differences between the application of one, two  
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47 or three cycles; extraction yields ranged between 65 and 74% (RSD = 2–7%,  $n = 3$ ). In this  
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51 investigation TTZ recovery was the lowest. It is concluded that for increased recovery it is  
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54 more important to use different extraction solvents rather than increasing the number of  
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60 extraction cycles. Due to its lean recovery TTZ was excluded from the study. Finally, one

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4 cycle (5 min extraction time) was selected to ensure the efficient extraction of CZ and DCZ  
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7 from eggs and plasma, so saving time for extraction.  
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## 10 Method validation

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14 Validation of the HPLC-UV method was done according to the requirements defined in the  
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18 guidelines of the European Commission Decision 2002/657/EC, which establishes the  
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21 performance criteria for analytical residue methods (EC, 2002). The partial validation  
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25 procedure includes determination of the specificity, recovery, precision in terms of  
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29 repeatability and within-laboratory reproducibility, decision limit ( $CC\alpha$ ), and detection  
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32 capability ( $CC\beta$ ) and robustness. The specificity of the method was assessed by testing a  
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36 total of 20 control eggs (yolk and albumen) and 10 plasma samples, to verify the absence  
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40 of potential interfering compounds. The best conditions were: mobile phase I ( $CH_3CN$ -  
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43  $H_2O$ ) with water at pH 4.5, analytical column SunFire, wave length 222 nm, flow rate 2.3  
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46 ml/min and using DCZ as the internal standard (5  $\mu g/ml$ ). Typical chromatograms for control  
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50 egg and plasma extracts and fortified egg and plasma samples are shown in [Figure 2A, B,](#)  
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54 [and Fig. 3.](#) The resulting chromatograms did not show evidence of interference, as no  
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57 detectable matrix peaks were eluted in the retention time window for the targets CZ and  
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60 DCZ. The limit of decision ( $CC\alpha$ ) is defined as the analyte concentration at, or above,

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4 which it can be concluded with an error probability ( $\alpha = 1\%$ ) that a sample is not compliant  
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7 ( $CC_{\alpha}$  is in the case of banned substances the lowest concentration level at which the  
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11 method can discriminate with a statistical certainty of  $1-\alpha$  whether the identified analyte is  
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14 present). Whereas the detection capability ( $CC_{\beta}$ ) for substances for which a 'not permitted  
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17 limit' has been established (as it is in the case of AC in eggs, for which no LMRL have  
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21 been defined), is the lowest concentration at which a method is able to detect truly  
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24 contaminated samples with an error probability ( $\beta = 5\%$ ) (European Commission, 2002).  
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28 Following these criteria specified in the Directive 96/23 Commission Decision 2002/657,  
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32  $CC_{\alpha}$  and  $CC_{\beta}$  were calculated independently for yolk, albumen and plasma from three  
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35 calibration curves, with analysis being performed on different days by spiking control egg  
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38 or plasma samples with CZ in the range 0.1–20  $\mu\text{g/g}$  and 5  $\mu\text{g/g}$  of the IS. These values  
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43 were calculated on the basis of multiple-spiked samples on each day. In the three matrices  
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46 the same values for  $CC_{\alpha}$  and  $CC_{\beta}$ , from 0.09  $\mu\text{g/g}$  and from 0.2  $\mu\text{g/g}$ , respectively, were  
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50 obtained. For quantitation of absolute recoveries during method optimisation and  
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53 validation, five-point matrix-matched calibration curves were prepared by fortifying control  
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57 egg and plasma extracts with CZ, within the range 0.1–20  $\mu\text{g/ml}$ , thus assuring a perfect  
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match between analysed samples and standard curves. Using the instrument conditions

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4 reported in the Materials and Methods, the calibration curves showed good linearity ( $R^2$  for  
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7 all curves were  $>0.999$ ). To evaluate the accuracy and intra-day repeatability of the  
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10 method, control egg samples, spiked with three concentration levels (1, 5 and 50  $\mu\text{g/g}$ ,  $n =$   
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13 12) were analysed using the optimised analytical method. The results are shown in [Table](#)  
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18 1. Recoveries of 64–74% were obtained for CZ, the target AC, at all levels of fortification  
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21 with RSD lower than 9%. Mean recoveries of the IS at the spiked concentration (5  $\mu\text{g/g}$ )  
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24 were  $(69 \pm 10)\%$ ; TTZ was excluded from the study because of its high retention time and  
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27 widened peak shape. To determine the linearity and the reproducibility of the whole  
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30 analytical method, control egg and plasma samples were fortified with the CZ in the range  
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33 0.1–20  $\mu\text{g/g}$  and 5  $\mu\text{g/g}$  DCZ was added as IS. Sample analysis using the procedure  
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36 described in the Materials and Methods was performed in triplicate on three different days.  
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43 The details of the method's validation are shown in [Table 2](#). No significant differences  
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46 (95% confidence limit) were observed between the calibration slopes obtained for the CZ  
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50 on different assay days, confirming the robustness of the analytical method. Therefore, the  
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53 reproducibility of the complete analytical procedure was evaluated from the percentage  
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56 recovery at each spiked level, using the regression curves and by interpolation of the peak  
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59 area values in the matrix-matched calibration curves. Excellent reproducibility was  
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3 achieved for CZ with mean recovery values of 68–74% and RSD lower than 11% in all  
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7 cases.  
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### 10 **Analysis of yolk and albumen and plasma samples**

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14 To further validate the method for the analysis using real samples, we analysed eggs  
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17 collected over a 21 d period from hens dosed with CZ (3 mg/kg BW) on 5 consecutive  
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20 days. A chromatogram of a contaminated egg sample collected on day 6 is shown in  
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25 Figure 2C. This indicates the presence of CZ in both the yolk and albumen. A new  
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28 undefined time-dependent peak was revealed with these samples. This could be a  
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32 metabolite of CZ but further analytical studies are needed to confirm this. Quantitation of  
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35 CZ in these samples was achieved by interpolation using the five-point calibration curves  
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38 made using control eggs spiked with CZ, as described in the Materials and Methods. The  
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42 results were used to construct depletion curves for CZ using yolk and albumen (Fig. 3), in  
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45 order to establish the MRL and the estimated withdrawal period. Error bars represent the  
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49 RSD for each daily sample set; these are related to differences in residue concentrations  
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53 in eggs collected from individual laying hens on a specific day. After a single dose of CZ,  
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57 none could be detected in yolks and albumens collected over a 10 d period. Some of the  
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60 samples were processed with an initial 10 g sample of the specific matrix, and

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3 reconstituted with 200  $\mu$ l of MeOH (concentration factor x 50), but even after this  
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7 procedure no residue could be detected in the two matrices.  
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11 Following multiple doses, CZ concentrations reached levels above LOQ within 2 d  
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14 of the start of the treatment, the highest values being detected in albumen and yolk, one  
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17 and three days, respectively, after the last administration of the drug. Thereafter, CZ  
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20 concentrations in albumen declined rapidly, within 3 d of the last treatment. The decrease  
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23 was much slower in yolk where the concentration was still above LOQ 10 d after the last  
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26 dose. The concentration of CZ in albumen was always much lower than in the yolk; the  
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29 maximum yolk CZ:albumen CZ ratio was about 10:1 three days after treatment ceased.  
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32 Although statistical analysis was not possible because of the limited number of samples,  
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35 the results in Figure 3 show a trend that indicates elimination of CZ in eggs (yolk and  
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38 albumen) and these are in agreement with data reported for the CZ analogue, DCZ  
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40  
41 (Mortier, *et al.*, 2005). These results indicate that CZ residues persist for a longer period in  
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44 yolk than in albumen, consistent with the fact that albumen is produced and secreted  
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47 within 24 h, while the turnover time for yolk is 6–8 d (Sauveur, 1988).  
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57 In order to evaluate the pharmacokinetics of CZ in laying hens, plasma  
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60 concentrations of the drug were measured after both single and multiple treatments. An

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3 equation, with one compartment of first order kinetics and with first order elimination  
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7 kinetics is shown in (Figure 4). The decay curve ( $R^2 = 0.92$ ) for a single dose of CZ is shown  
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10 in Figure 5 and for multiple doses of CZ the pattern ( $R^2 = 0.92$  and  $0.95$ ) the pattern is shown in  
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12  
13 Figure 6. The plasma concentrations derived from the three pools, each from sera of 4 birds, for  
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15 both single and multiple dose treatments are provided (Tables 3 and 4); due to the limited number  
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17 of samples any conclusion from these data would be complete speculation. However, after a single  
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19 dose (Figure 5) plasma CZ appears to achieve a higher concentration compared with multiple doses  
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21 (see Table 5). Following both methods of administration the constant for the absorption phase ( $K_{01}$ )  
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23 was higher than the constant of the elimination phase ( $K_{10}$ ), suggesting that the rate of absorption is  
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25 higher than that for elimination. Although these values were higher after single rather than multiple  
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27 doses, their ratio ( $K_{01}/K_{10}$ ) was the same for both treatments. The half lives for the elimination  
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29 phase after single and multiple doses were 7.4 and 16.4 h, respectively, suggesting predicted  
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31 washout periods of about 50 and 115 h, respectively. The wide divergence in the clearance value  
32  
33 (CL) is substantially down to the different methods of dosing. After a single dose, CZ seems to  
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35 reach a high plasma concentration compared with multiple doses (Table 5). This could be due to the  
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37 limited number of samples, because plasma concentration data were derived from only three pools  
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39 (each from 4 hens) with both single and multiple dose treatments (Tables 3-4), consequently any  
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41 pharmacokinetic explanation remains speculation.  
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49 A single dose of 2.5 mg CZ /kg has been shown to be effective against coccidiosis in  
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51 pigeons, although it is not known how this is brought about (Vercruysse, 1990). If the AC  
52  
53 properties of CZ can be demonstrated for laying hens, CZ could be useful as an AC drug for such  
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55 birds because of the very low residues in eggs (undetected using the present method). On the other  
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57 hand after multiple doses, CZ reached steady state after the third dose (Figure 6) and its residues in  
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59 eggs yolk persisted for several days.  
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3 In conclusion, we report for the first time the HPLC detection of CZ in yolk, albumen and  
4 plasma from laying hens. The method provides good recovery without the need for further clean-up  
5 steps. The short time required for the analysis leads us to propose this method for routine use. The  
6 decision limit ( $CC\alpha$ ) and detection capability ( $CC\beta$ ) of the method (0.09  $\mu\text{g/g}$  and from 0.2  $\mu\text{g/g}$ ,  
7 respectively) were found to be the same in three different matrices, indicating that the method could  
8 be useful for determining CZ residues in contaminated eggs and also for screening for illegal use of  
9 CZ in laying hens. Although other studies concerned with the efficacy of CZ as an AC for this  
10 species and more sensitive methods of detection will be necessary, the residue in the eggs after a  
11 single dose of 3 mg CZ/kg BW, appears to be negligible. However, pharmacokinetic and residual  
12 data after multiple doses of CZ show that drug does accumulate in yolk, albumen and plasma.  
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## Figure captions

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10 **Figure 1.** *Molecular structures of toltrazuril (A), diclazuril (B) and clazuril (C).*

11 **Figure 2.** *A) Chromatographic curves from blanks of albumen (I) solid line, yolk (II) dotted line*  
12 *and plasma (III) dashed line.*

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17 *B) Chromatographic curves from fortified samples (CZ and DCZ 5  $\mu\text{g/g}$ ) for albumen (I)*  
18 *solid line, yolk (II) dotted line and plasma (III) dashed line.*

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22 *C) Chromatographic curves from naturally-contaminated samples of albumen (I) solid*  
23 *line, yolk (II) dotted line and plasma (III) dashed line.*

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27 **Figure 3.** *CZ concentrations ( $\mu\text{g/g}$ ) in yolk ( $\square$ ) and albumen ( $\Delta$ ) following multiple administration*  
28 *( $n=5$ ) of drug (3 mg/kg BW day) in laying hens. Day zero is the day of starting treatment. Error*  
29 *bars represent the standard deviation.*

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34 **Figure 4.** *Equation, one-compartment with first-order input and output, which best describes*  
35 *plasma concentrations vs time data.*

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39 **Figure 5.** *Mean plasma concentrations of CZ following a single oral dose (3 mg/kg BW) using*  
40 *pools of plasma each from three laying hens ( $n=4$ ). Solid line predicted concentrations, dotted line*  
41 *observed concentrations.*

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46 **Figure 6.** *Mean plasma concentrations of CZ following a multiple ( $n=5$ ) oral dose (3 mg/kg BW*  
47 *day) using pools of plasma each from laying hens ( $n=4$ ). Solid line predicted concentrations,*  
48 *dotted line observed concentrations.*

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55 **Table 1.** *Intra-day recoveries (%) and precision (RSD, %) obtained for the analysed CZ after*  
56 *extraction of egg (yolk and albumen) and plasma samples ( $n=3$ ) spiked at different concentration*  
57 *levels.*

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3 **Table 2.** *Inter-day mean recovery (n=5) and precision (RDS%) data for the determination of CZ in*  
4 *fortified egg (yolk and albumen) and plasma samples, performed on three different days.*  
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8 <sup>a</sup>*Average calibration equation. Uncertainty values calculated at a 95% confidence limit; 5 data*  
9 *points (n=3). C = CZ concentration (µg/ml); IS: DCZ (5 µg/ml).*  
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12 **Table 3.** *Mean plasma concentrations of CZ following a single oral dose (3 mg/kg BW) in three*  
13 *pools of laying hens (n=4).*  
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16  
17 **Table 4.** *Mean plasma concentrations of CZ following a multiple (n=5) oral dose (3 mg/kg BW*  
18 *day) in three pools of laying hens (n=4).*  
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21  
22 **Table 5.** *Pharmacokinetic parameters, mean ± SD, of CZ (3 mg/kg) following oral single and*  
23 *multiple (n=5) dose.*  
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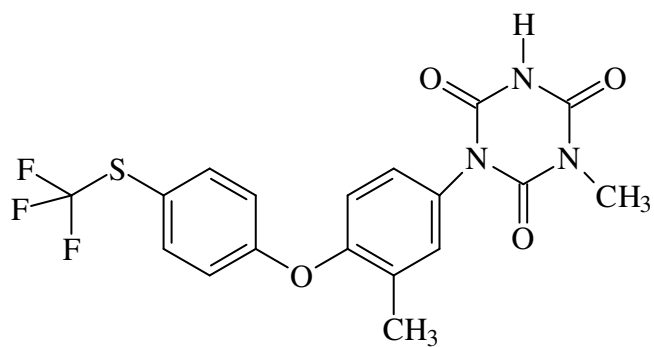
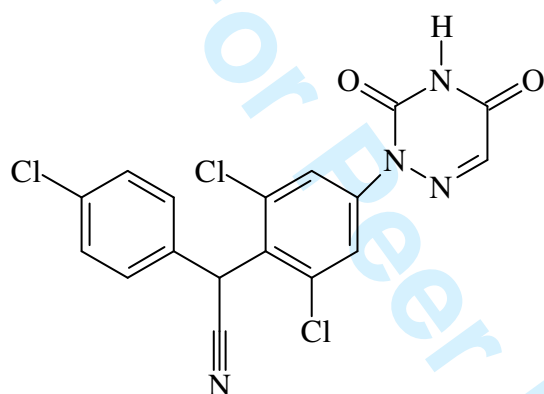
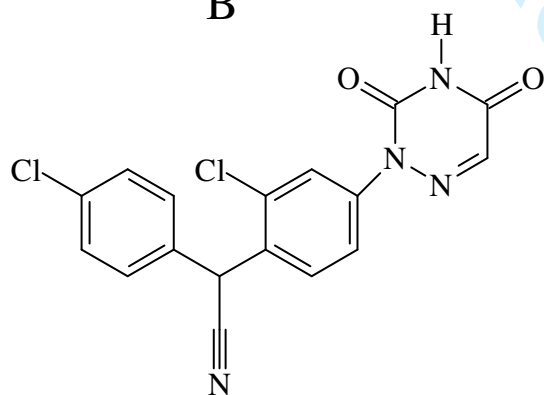
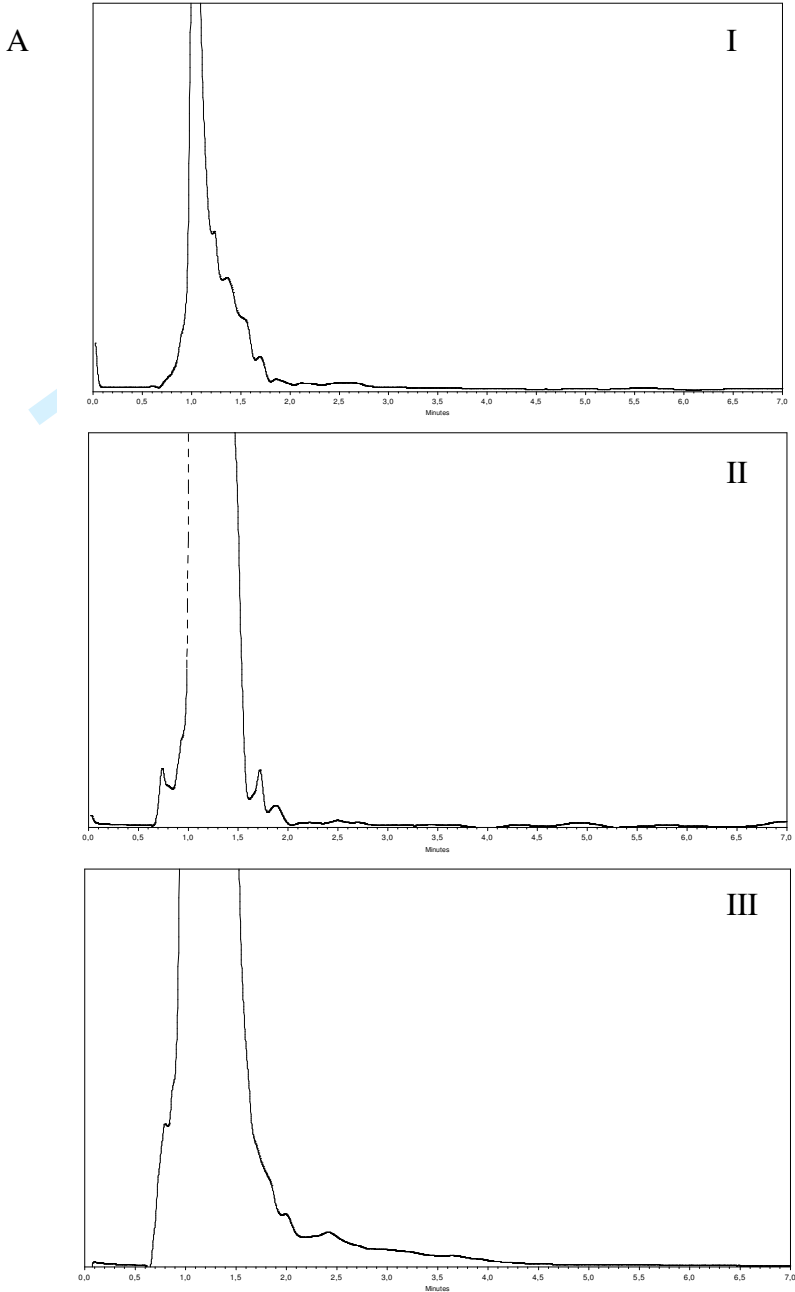
**A****B****C**

Figure 1 – Molecular structures of toltrazuril (A), diclazuril (B) and clazuril (C).

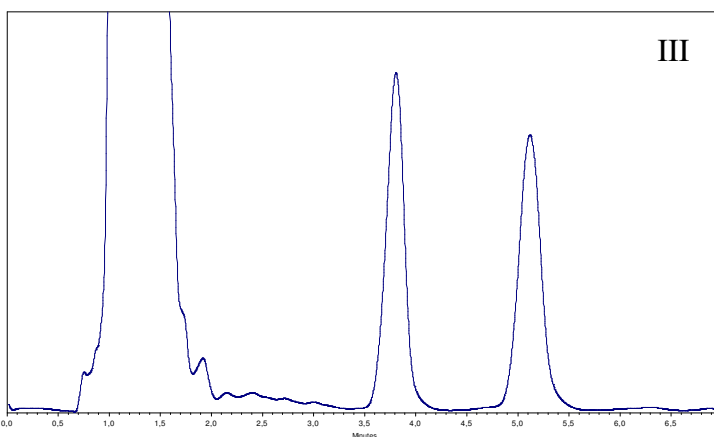
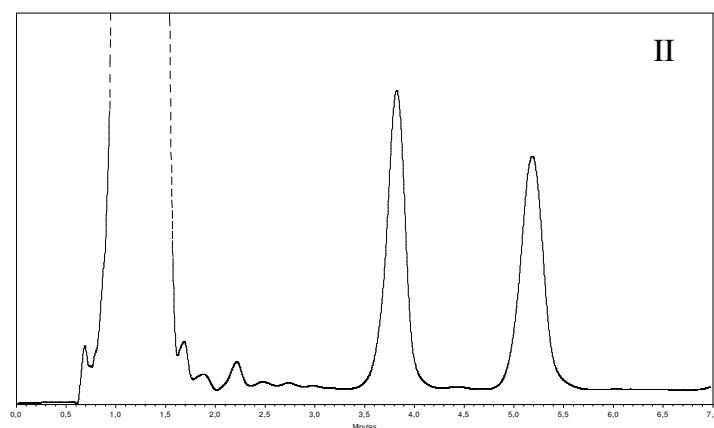
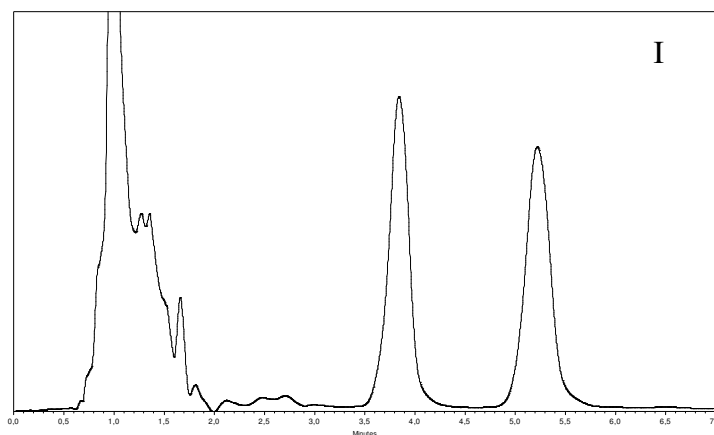


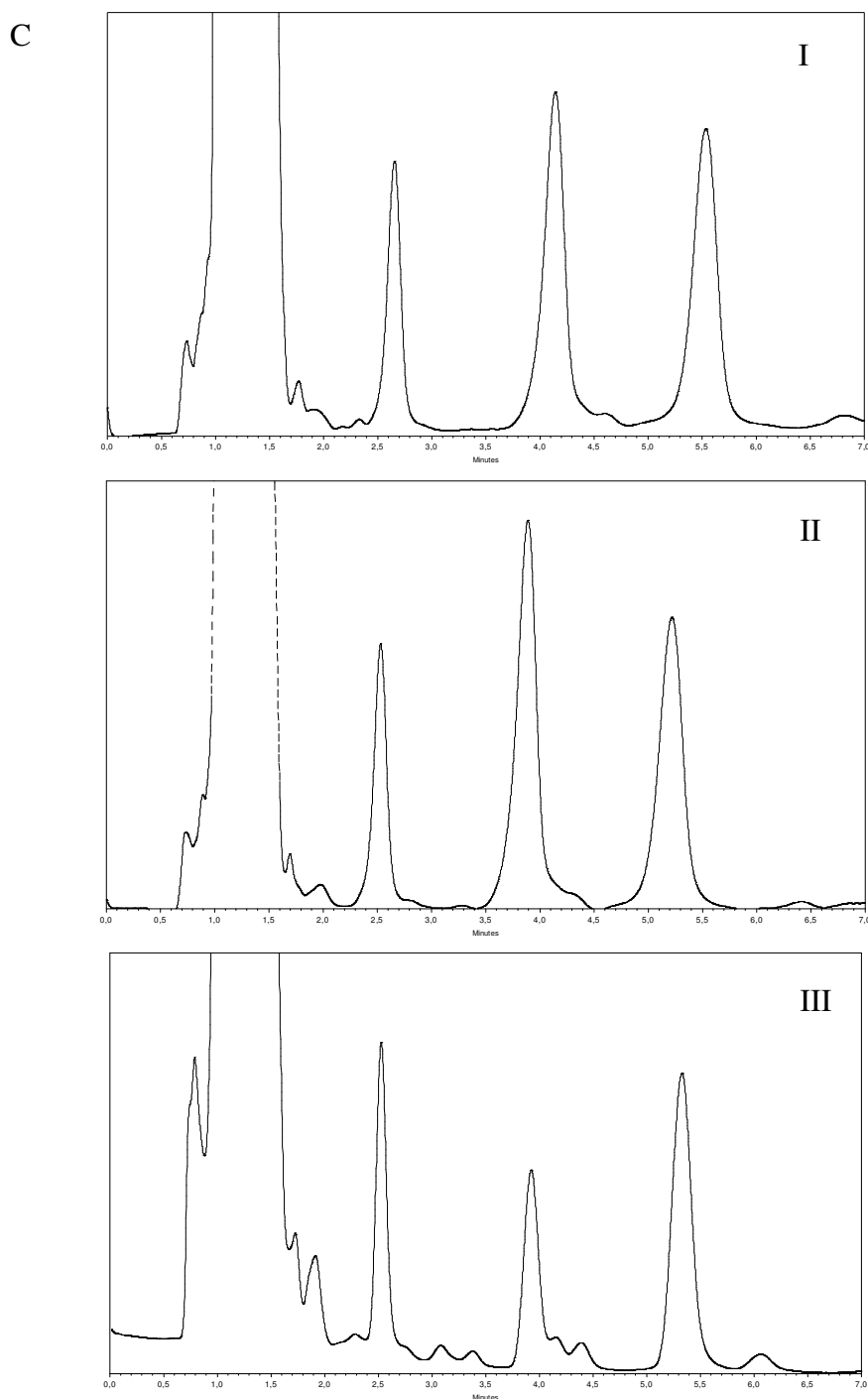
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51 Figure 2 – A) Chromatographic curves from blanks of albumen (I) solid line, yolk (II) dot line and  
52 plasma (III) dash line.

53 B) Chromatographic curves from fortified sample (CZ and DCZ 5 µg/g) of albumen (I)  
54 solid line, yolk (II) dot line and plasma (III) dash line.

55 C) Chromatographic curves from incurred samples of albumen (I) solid line, yolk (II) dot  
56 line and plasma (III) dash line.  
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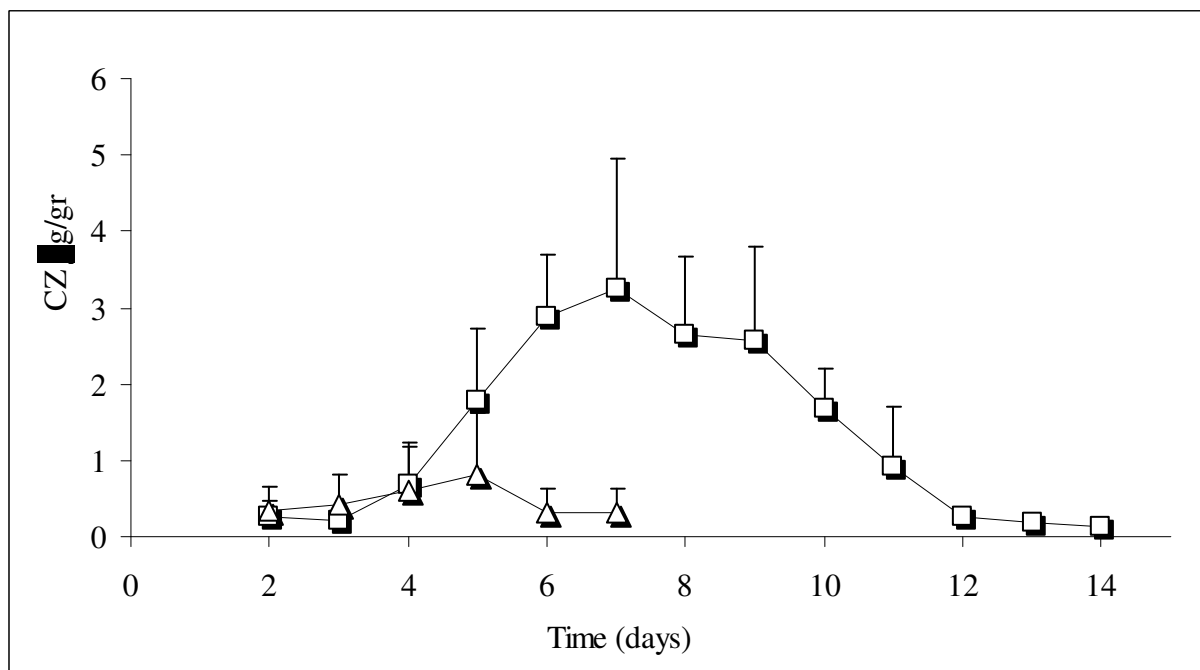
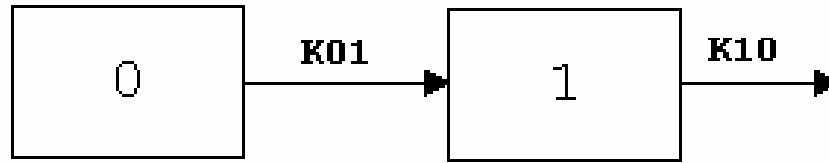


Figure 3



$$C(T)=D K_{01}/V/(K_{01}-K_{10}) [\text{EXP}(-K_{10} T) -\text{EXP}(-K_{01} T)]$$

Figure 4 – Equation, one-compartment with first-order input and output, that best describes plasma concentrations vs time data.

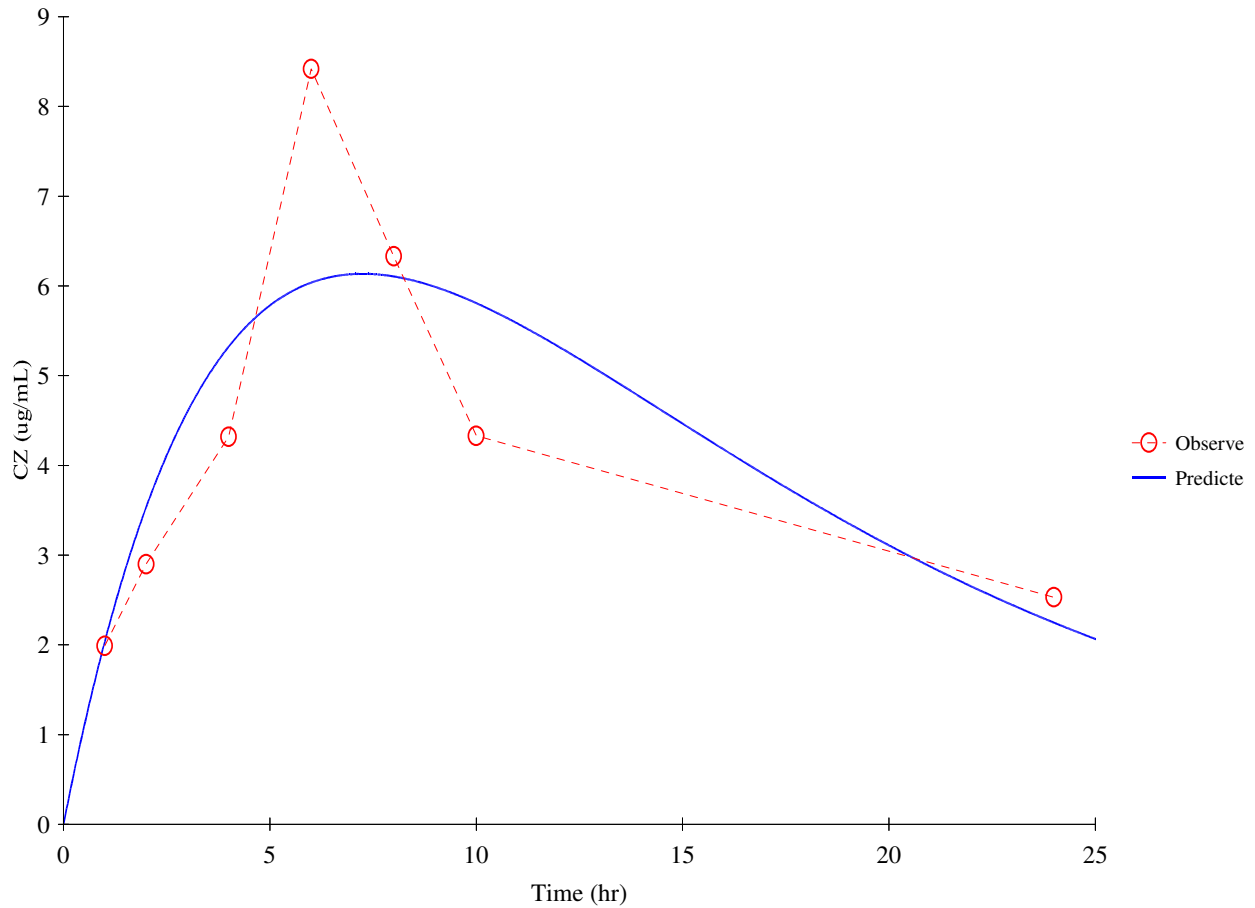


Figure 5

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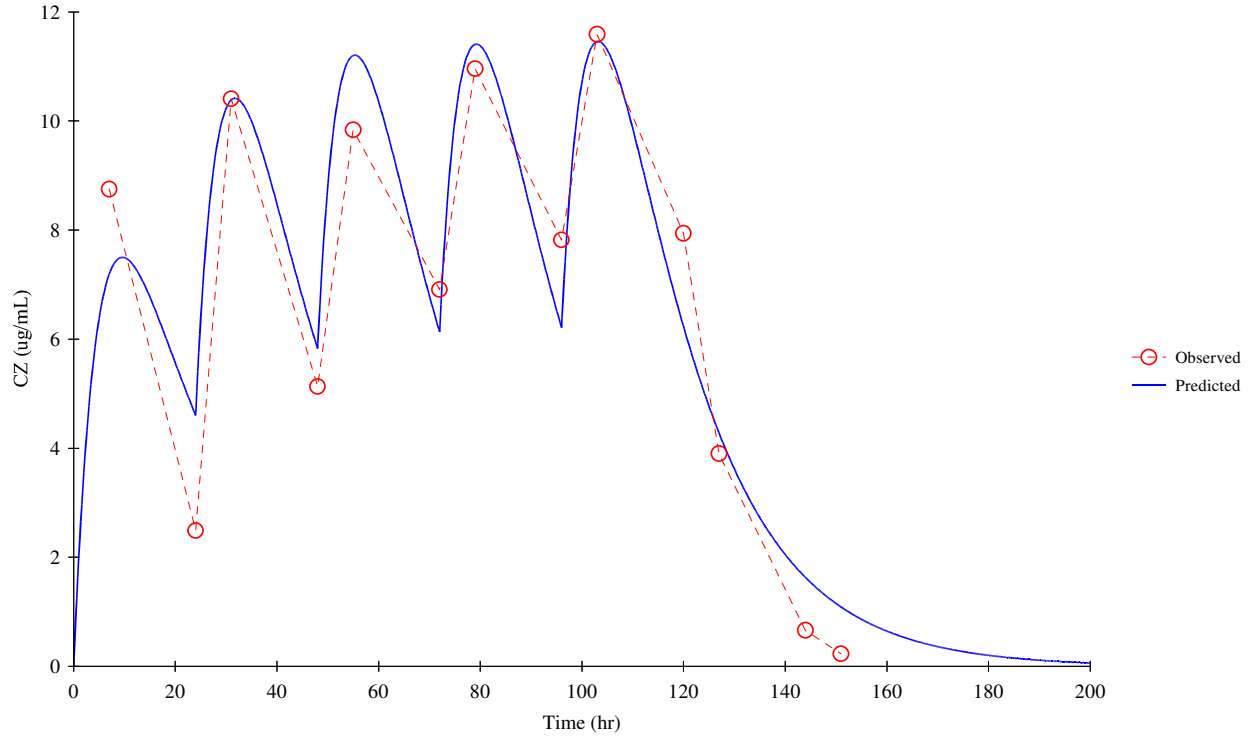


Figure 6

Review Only

Drug	1 µg/mL		5 µg/mL		50 µg/mL		Matrix
	R%	RSD%	R%	RSD%	R%	RSD%	
CZ	69	9	74	6	70	7	Yolk
	64	8	72	6	71	8	Albumen
	69	8	74	6	74	7	Plasma

Table1 - Intraday recoveries (%) and precision (RSD%) obtained for the analyzed CZ after extraction of egg (yolk and albumen) and plasma samples (n=3) spiked at different concentration levels.



Drug	Spiked amount ( $\mu\text{g/g}$ )	R%	RDS%	Matrix
CZ $A_{\text{CZ}}/A_{\text{IS}} = (115 \pm 11) 10^{-4} C + (0.03 \pm 0.02)^{\text{a}}$	0.1	66	9	Yolk
	1	72	6	
	5	68	10	
	10	74	7	
	20	71	6	
-----				
CZ $A_{\text{CZ}}/A_{\text{IS}} = (230 \pm 49) 10^{-4} C + (0.04 \pm 0.01)^{\text{a}}$	0.1	69	9	Albumen
	1	72	8	
	5	69	9	
	10	74	11	
	20	70	6	
-----				
CZ $A_{\text{CZ}}/A_{\text{IS}} = (99 \pm 15) 10^{-4} C + (0.05 \pm 0.02)^{\text{a}}$	0.1	67	8	Plasma
	1	69	10	
	5	74	9	
	10	74	8	
	20	71	9	

Table 2 - Interday mean recovery (n=5) and precision (RDS%) data for the determination of CZ in fortified egg (yolk and albumen) and plasma samples, performed on three different days. <sup>a</sup>Average calibration equation. Uncertainly values calculated at a 95% confidence limit; five data points (n=3). C = CZ concentration ( $\mu\text{g/mL}$ ); IS: DCZ (5  $\mu\text{g/mL}$ ).

<u>Time (hours)</u>	<u>CZ (<math>\mu\text{g/ml}</math>)</u>	<u>SD</u>
1	1.99	1.87
2	2.90	0.21
4	4.32	0.37
6	8.42	0.54
8	6.33	0.16
10	4.33	1.85
24	2.53	0.23

Table 3 - Mean plasma concentrations of CZ following a single oral dose (3 mg/kg BW) in 3 pools of laying hens (n=4)

<u>Time (hours)</u>	<u>CZ (µg/ml)</u>	<u>SD</u>
7	8.75	0.22
24	2.49	0.12
34	7.83	0.55
48	5.13	0.45
55	9.84	2.11
72	6.91	0.40
79	10.96	3.48
96	7.82	1.15
103	11.59	2.37
120	7.94	2.02
127	3.90	0.36
144	0.66	0.21
151.00	0.23	0.04

Table 4 - Mean plasma concentrations of CZ following a multiple (n=5) oral dose (3 mg/kg BW day) in 3 pools of laying hens (n=4)

Parameters	Single dose	Multiple dose
R <sup>2</sup>	0.92	0.95
AUC (h µg/ml)	129.20±43.07	223.81±13.67
K <sub>01</sub> (1/h)	3.41	0.34
K <sub>10</sub> (1/h)	0.60	0.06
K <sub>01</sub> t <sub>1/2</sub> (h)	3.56±1.91	1.96±0.68
K <sub>10</sub> t <sub>1/2</sub> (h)	7.40±4.33	16.42±4.24
CL (ml/h/kg)	23.21±5.65	13.43E <sup>-02</sup> ±8.23E <sup>-04</sup>
Tmax (h)	7.25±2.27	6.83±4.21
Cmax (µg/ml)	6.14±0.84	7.06±0.74

Table 5 - Pharmacokinetic parameters, mean ± SD, of CZ (3 mg/kg BW) following single and multiple (n=5) dose.