

Assessment of enrofloxacin and ciprofloxacin accumulation in pig and calf hair by HPLC and fluorimetric detection

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Abstract Enrofloxacin is a synthetic bacteriostatic administered in veterinary therapy. It can also be used illegally as a growth promoter to enhance feed efficiency and weight gain. This practice is banned in several countries due to its potential negative effects on the environment and human health. A suitable method for extracting and quantifying enrofloxacin (ENR) and its main metabolite ciprofloxacin (CPR) in cattle and pig hair by high-performance liquid chromatography–fluorimetric detection (HPLC–FLD) had been proposed. ENR and CPR were extracted from hair samples with methanol acidified with trifluoroacetic acid for 24 h at 70 °C. The extracts were evaporated and redissolved in the mobile phase before injection. This simplified procedure enabled the detection of both CPR and ENR at ng g^{-1} levels (limit of detection 4–5 ng g^{-1}) without further purification. Detectable residues of ENR were found in calf and pig hairs after the pharmacological treatment was started. Mean concentrations of quinolone (ENR, CPR) in contaminated hairs ranged from 20 to 2,518 ng g^{-1} in calves and from 152 to 1,140 ng g^{-1} in pigs. Hair pigmentation enhanced quinolone accumulation significantly. Hair analysis seems to increase the time window available for the retrospective detection of illegal ENR administration compared to edible tissue analysis.

Keywords Drug monitoring/drug screening · Bioanalytical methods · Fluorescence/luminiscence · HPLC

Introduction

Fluoroquinolones are an important group of synthetic bacteriostatics used in human and veterinary therapy to prevent and treat respiratory, urinary and enteric diseases. Their misuse (as well as the misuse of other antibiotics) as growth-promoting agents to enhance livestock productivity is believed to be one of the factors responsible for the appearance of microbial resistance [1, 2]. For this reason, both the EU and the USA—where quinolones have been used as growth promoters for a long time—have restricted or prohibited their application to enhance weight gain in livestock production [1]. More recently, the FDA announced a ban on the distribution or use of enrofloxacin to treat bacterial infections in poultry [3].

More generally, many countries protect consumer health from possible drug residues in foods by setting, if necessary, tolerable concentrations of drug residues that are said to present virtually no toxicological risk to health, and surveillance plans have been implemented to check for compliance with the relevant directives [4, 5]. As an example, the maximum residue level (MRL) established within the EU for the quinolone enrofloxacin (ENR), taking into account its primary metabolite ciprofloxacin (CPR), which is produced by oxidative dealkylation in liver [6], ranges from 100 to 300 ng g^{-1} in edible tissues [4, 6, 7].

For several years hair analysis has been considered to be a useful technique for increasing the time window for the retrospective detection of veterinary drug administration, and for enhancing the effectiveness of surveillance plans [8, 9]. The accumulation of veterinary drugs in livestock hair has already been established for various anabolics (β -agonists, hormonal compounds) and for several antibiotics in cattle and horses [8–10]. Quinolones have been detected in human hair after alkaline digestion (NaOH at 80 °C) and

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liquid–liquid purification [11–17], as well as in horsehair after acidic extraction (trifluoroacetic acid 0.2 M at 70 °C) followed by SPE purification [18]. Many authors have employed fluorimetric detection after chromatographic separation due to the natural fluorescence of these compounds [19].

However, there is a general lack of knowledge about the mechanisms by which drugs are incorporated into the hair structure, as well as the multiple factors that can influence the pharmacokinetics and the pharmacodynamics in such tissue [20–22]. Furthermore, there is a lack of specific studies in the scientific literature on the accumulation of quinolones in hair of food-producing animals, and no data have been published that can clarify the relationship between the residual quinolone levels in hair and those in edible tissues of the same animal, which would be useful for the practical application of hair analysis.

The first aim of this study was therefore to develop a simplified analytical protocol to extract and quantify ENR and CPR in cattle and pig hair by HPLC–FLD for screening purposes. The role of hair pigmentation in drug accumulation after its administration and a comparison of the drug residue levels in hair, liver and muscle from the same animals are also considered and discussed here.

Materials and methods

Reagents

Enrofloxacin and ciprofloxacin (ACS grade), tris(2-carboxyethyl)phosphine hydrochloride (TCEP) 0.5 M solution, ammonium hydroxide, sodium hydroxide, oxalic acid, diethylmalonic acid, and trifluoroacetic acid (TFA) 25% solution in water were purchased from Sigma-Aldrich (Madrid, Spain). Methanol and acetonitrile (liquid chromatography grade), dichloromethane and nitric acid 65% (v/v) were from Merck (Darmstadt, Germany). Ultrapure water was obtained using an E-pure system (Barnstead Thermolyne, Dubuque, IA, USA).

Acidified methanol (TFA 0.2 M) was prepared by mixing 0.5 mL of the TFA 25% solution with 99.5 mL of methanol, while aqueous TFA 0.2 M was prepared by diluting 0.5 mL of the TFA 25% solution up to 100 mL with ultrapure water.

Two different veterinary drugs obtained from local stores were employed for the pharmacological treatments: an injectable solution (Hipralona ENRO-I containing 50 mg mL⁻¹ enrofloxacin, from HIPRA, Girona, Spain), and an oral solution (Colmyc, containing 100 mg mL⁻¹ enrofloxacin, from S.P. Veterinaria S.A. Laboratories, Tarragona, Spain).

Single standard stock solutions [100 mg L⁻¹] for each quinolone were prepared by dissolving 10 mg of pure standards in 100 mL of 0.01 M nitric acid. Chromatographic standard solutions [0.01, 0.1, 1 and 10 mg L⁻¹] were prepared weekly by diluting of standard stock solutions with 0.01 M oxalic acid: ACN [88:12] (v/v), and these were stored at 41 °C.

Animal treatment

Six Friesian calves were reared to eight months at the IRTA experimental farm (Prat de Llobregat, Spain). In the final five weeks before slaughter, they were divided into three groups, and maintained in separate stalls during the last growth phase. The first group (controls) did not receive any veterinary drug containing quinolones. The second group (WP) was supplied, complying with a withdrawal period of 28 days, with 7.5 mg ENR kg⁻¹ live weight (daily intramuscular dose of 20 mL Hipralona ENRO-I solution per animal for three days) (Fig. 2). The third group (NoWP) received the same treatment with Hipralona ENRO-I, just a week before slaughter, without observing any withdrawal period.

Four Landrace pigs were also reared in the same experimental farm for up to three months. During the last four weeks before slaughter, a group of two animals (NoWP) received a total dose of 42 mg ENR kg⁻¹ live weight (daily oral dose of 1.5 mL of Colmyc mixed with feed for 28 days). The other group of animals received no medication containing any quinolone (controls).

Sampling

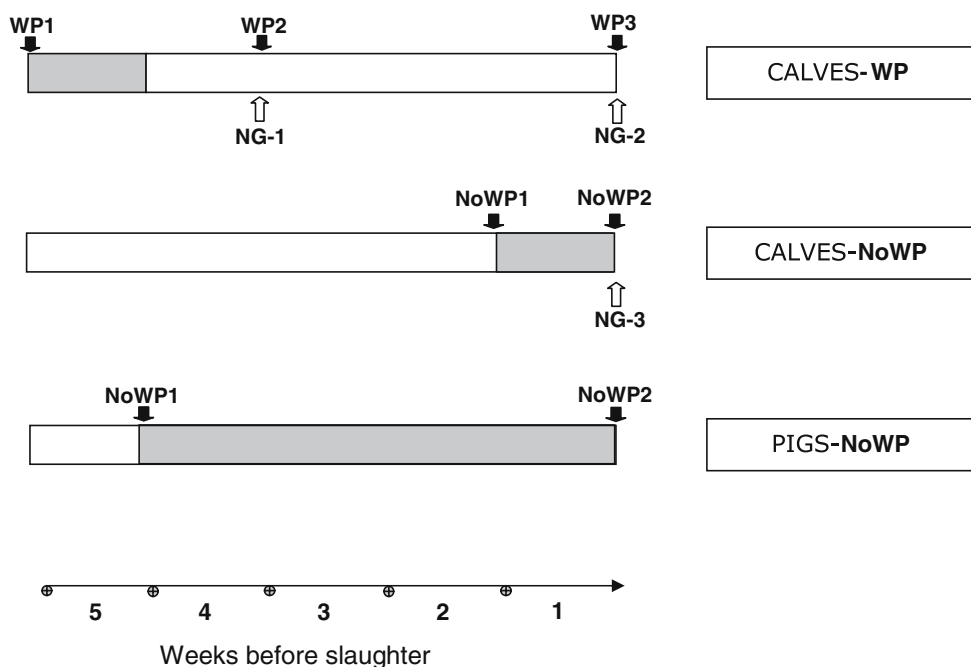
Pigmented and unpigmented hair samples were taken from all treated calves before (WP1, NoWP1) and after (WP2, WP3, NoWP2) the beginning of the pharmacological treatments. From these animals, additional samples (NG-1, NG-2, NG-3) corresponding to newly growing hair (NG) were taken sequentially from the same areas as the first sampling areas (WP1, NoWP1) (Fig. 1).

Pigmented and unpigmented hair samples were taken separately from the corresponding homogeneous areas of the animals (white or black). In the case of treated pigs, unpigmented hair samples were taken before the treatment (NoWP1) and four weeks after the first ENR administration, just before the slaughter (NoWP2).

Hair samples were taken from the upper area of the neck (to limit external contamination) with a hair clipper (Arco 1854, Moser, Germany), at a cut height of 1 mm. Hair samples were also obtained from all control animals just before the beginning of the experiment and before slaughter.

A total of 32 hair samples (8 samplings × 2 pigmentations × 2 animals) from treated calves and 8 hair samples from control

Fig. 1 Pharmacological treatment and sampling scheme for treated animals. *WP* (withdrawal period); *NoWP* (without withdrawal period); *NG* (newly growing hair). Grey zones indicate treatment with ENR. Arrows indicate different sampling points



calves (2 samplings \times 2 pigmentations \times 2 animals) were obtained. Four hair samples from treated pigs (2 samplings \times 2 animals) and 4 hair samples from control pigs (2 samplings \times 2 animals) were taken. After slaughter, samples of liver and muscle (diaphragm) were also taken from all animals. All of the tissues (hair, liver and muscle) were placed in aluminum bags and maintained at $-20\text{ }^{\circ}\text{C}$ until analysis.

Hair pre-treatment

Hair samples were rinsed three times with ultrapure water, cut finely with scissors (to $\sim 2\text{ mm}$), dried at $37\text{ }^{\circ}\text{C}$, and stored in a desiccator as previously described [23]. A hair “pool sample” (4.00 g) was also obtained by mixing pigmented calf hair samples from animals that had undergone different treatments.

Quinolone extraction from hair

TCEP 25 mM, NH_4OH 0.2 M and MeOH were tested as ENR extractants in preliminary trials, but because of their poor results they were not considered any further. However, two extractants containing TFA were investigated further.

Aliquots of 100 mg of the hair “pool sample” were extracted with 4 mL of acidified (TFA 0.2 M) methanol or aqueous TFA 0.2 M for 1, 16, 24 and 48 h at $70\text{ }^{\circ}\text{C}$. Once extracted, the samples were filtered through a paper filter, diluted to 10 mL with the mobile phase, filtered and analyzed by HPLC-FLD.

Once the extraction conditions were optimized, all of the hair samples were extracted with 4 mL of acidified methanol for 24 h at $70\text{ }^{\circ}\text{C}$. After filtration, the extracts

were evaporated under a stream of nitrogen, reconstituted with the mobile phase (500 μL) and filtered through a nylon membrane filter (0.45 μm porosity). Recoveries for ENR and CPR were evaluated by fortifying control hair samples at 0.5 and $1\text{ }\mu\text{g g}^{-1}$ with 50 or 100 μL of standard solution (1 mg L^{-1}). Once spiked, the hair samples were homogenized by vortexing them and then equilibrated at ambient temperature for 10 min before performing the extraction procedure.

Quinolone extraction from liver and muscle

Liver and muscle from cattle and pigs were analyzed as previously described in order to detect residues of both compounds in broiler tissues [24]. Briefly, 2.0 g of minced tissue were weighed in a 30 mL centrifuge tube and then added to 1.5 mL of 0.25 M diethylmalonic acid, buffered at pH 7.00, and 10 mL of dichloromethane. This mixture was mixed vigorously and centrifuged at 2,000 g for 10 min (Beckman J2-MC, Fullerton, CA, USA). The dichloromethane layer was taken and the extraction was repeated. Dichloromethane fractions were combined, added to 2 mL of 1 M NaOH and centrifuged as above. One milliliter of the aqueous alkaline extract was mixed with 1 mL of oxalic acid 0.5 M, filtered through a 0.45 μm nylon filter, and injected into the HPLC-FLD system. Recovery assays were created by fortifying control tissue samples at $0.5\text{ }\mu\text{g g}^{-1}$ with 100 μL of standard solution (10 mg L^{-1}).

Once spiked, the tissue samples were homogenized by vortexing them, and then they were equilibrated at ambient temperature for 10 min before performing the extraction procedure.

HPLC analysis

Chromatographic analyses were carried out on a 1100 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump and an HP (Palo Alto, CA, USA) 1046A fluorescence detector ($\lambda_{\text{exc}}=280$ nm; $\lambda_{\text{em}}=444$ nm). Chromatographic separation was performed on a Luna RP-C₈ (250 mm×4.6 mm id.) column (Phenomenex, Torrance, CA, USA) at a flow rate of 1 mL min⁻¹. A binary gradient elution was performed, varying the composition of the mobile phase linearly from 88% A (oxalic acid 0.01 M) and 12% B (acetonitrile) to 80% A and 20% B at 18 minutes. One hundred microliters of hair extracts and 50 μ L of liver and muscle extracts were injected. Each sample was analyzed in duplicate.

ENR and CPR peaks were identified by comparing their retention times with those of the standard solutions. Quantification was performed using an external calibration curve created by injecting 0.1, 1, 10, 50 and 100 ng of both CPR and ENR in duplicate and by taking into account recovery values and dilution.

Statistical analyses

Analysis of variance (ANOVA) was used to test hypotheses about differences between the ENR and CPR accumulated in contaminated pigmented and unpigmented cattle hairs (6 samplings×2 pigmentations×2 animals; $n=24$ sam-

ples). Control samples and negative samples (<LOD) were not considered.

Sampling and hair pigmentation were considered to be independent variables, while ENR or CPR residual concentrations were the dependent variables. Replicates of analyses ($n=2$) were also taken into account. The statistical treatment was carried out with the software package Statistica 5.0 (StatSoft, Inc., Tulsa, OK, USA).

Results and discussion

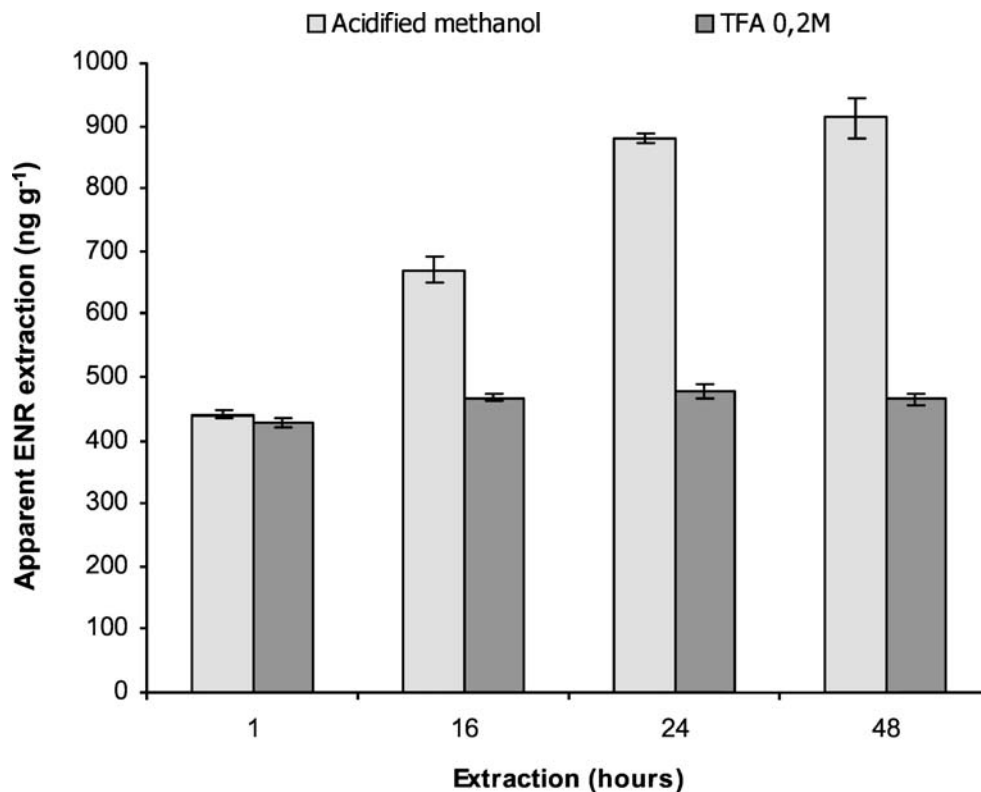
Quinolone extraction from hair samples

Temperatures of less than 70 °C, as well as the use of MeOH and TCEP (25 mM in water) as extracting phases, were discarded because low apparent ENR extractions were observed. The use of alkaline digestion (0.2 M NH₄OH) gave good ENR apparent extractions but produced complex extracts which would need a further purification step and so this approach was discarded too.

Therefore, taking into account the results of Dunnett et al. [18], only two extractant solutions, both containing TFA, were investigated further. For our experimental conditions, acidified methanol gave better apparent ENR extraction than aqueous TFA 0.2 M (Fig. 2), especially for extraction times of between 24 and 48 hours.

Acidified methanol also gave clear extracts which could be easily concentrated without the need for further

Fig. 2 Time course of ENR extraction from the pigmented hair “pool sample” using acidified methanol and aqueous TFA 0.2 M at 70 °C. Concentrations are expressed as mean values ($n=4$) \pm SD



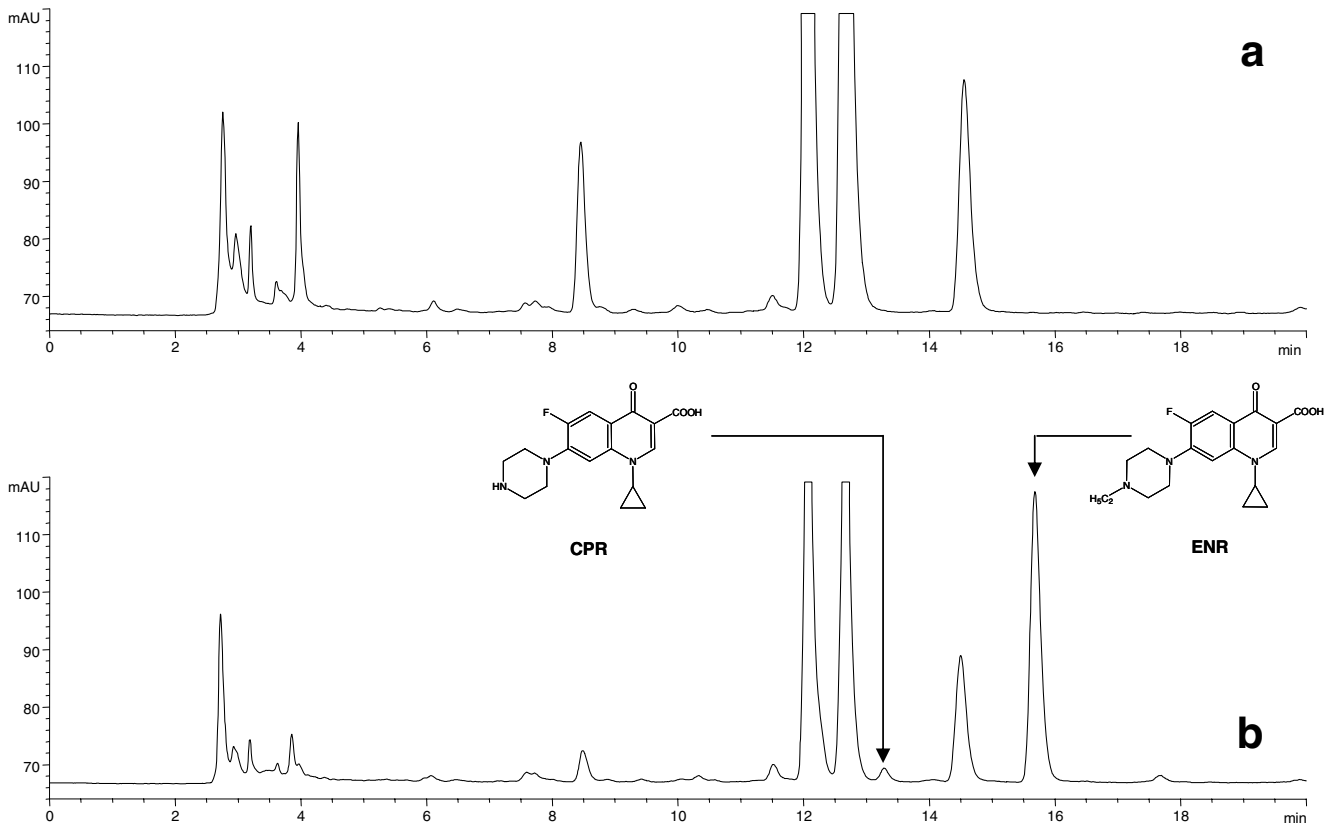


Fig. 3a, b Chromatographic profile corresponding to pigmented calf hair extracts. **a** Control, **b** treated samples

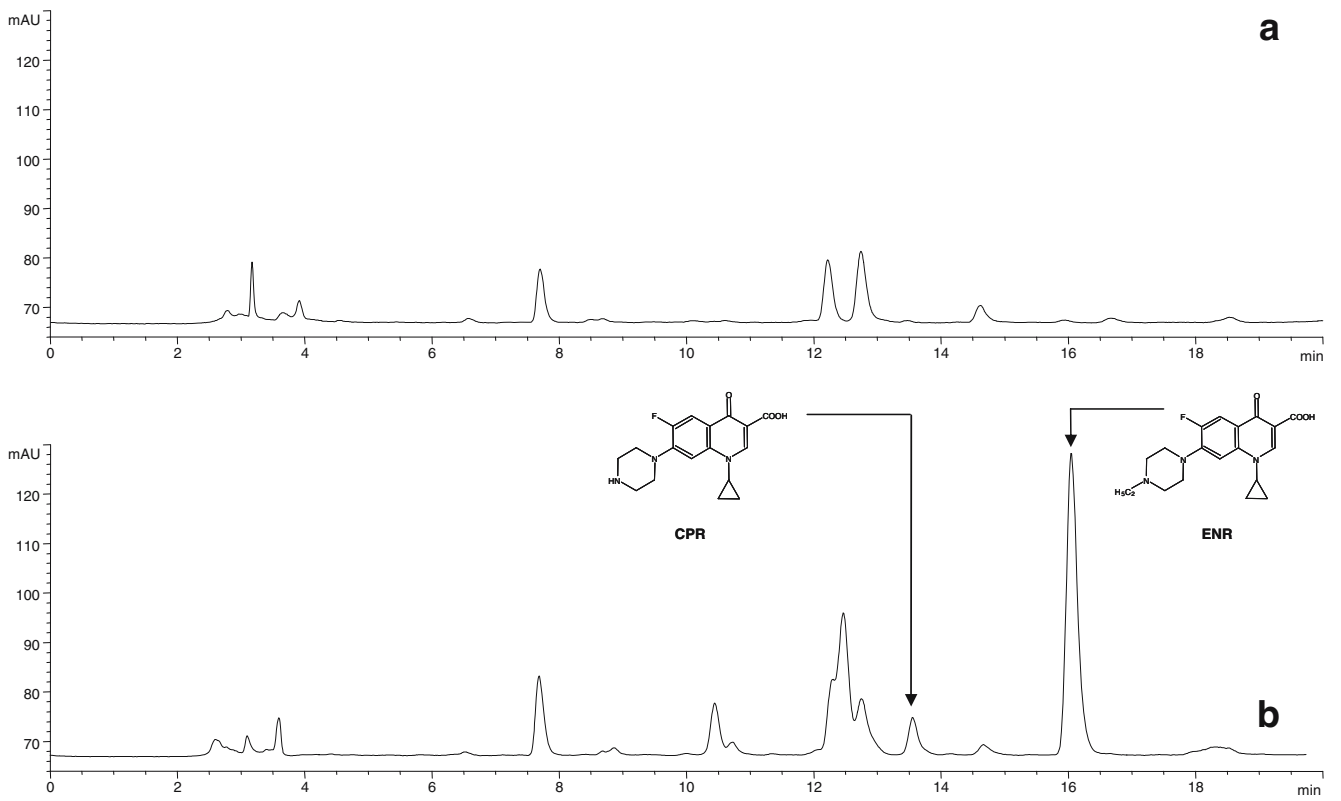


Fig. 4a, b Chromatographic profile corresponding to unpigmented pig hair extracts. **a** Control, **b** treated samples

Table 1 Recoveries, limits of detection (LOD) and limits of quantification (LOQ) for the analysis of hair samples

| Compound | Animal | Spiking level ($\mu\text{g g}^{-1}$) | Accuracy | | Sensitivity | |
|----------|--------|---|-------------------|------|------------------------|------------------------|
| | | | Recovery (%) | | LOD | LOQ |
| | | | Mean | SD | (ng g^{-1}) | (ng g^{-1}) |
| ENR | Calf | 0.5 | 62.4 ^a | 3.0 | 4 | 14 |
| | | 1.0 | 64.8 ^b | 4.8 | | |
| | Pig | 0.5 | 77.1 ^a | 10.6 | 4 | 12 |
| | | 1.0 | 69.1 ^c | na | | |
| CPR | Calf | 0.5 | 62.6 ^a | 1.4 | 5 | 16 |
| | | 1.0 | 63.8 ^b | 6.1 | | |
| | Pig | 0.5 | 76.0 ^a | 4.5 | 4 | 15 |
| | | 1.0 | 65.2 ^c | na | | |

^a $n=6$ ^b $n=3$ ^c $n=1$

n.a., not available

purification steps. Considering these results, the final extraction conditions applied to analyze all the hair samples were acidified methanol and 24 hours of contact at 70 °C.

HPLC analyses

The chromatographic conditions were adjusted to separate and detect both ENR and CPR in hair extracts without the need for purification steps. Figures 3 and 4 show typical chromatograms obtained from control and contaminated cattle and pig hairs. The response was linear for injected

amounts of 0.01–100 ng ENR and CPR ($r^2 > 0.999$), and peak symmetry was good for both compounds. Complete analysis of hair extracts was possible within 20 minutes, with retention times of 13.4 ± 0.1 min observed for CPR and 15.8 ± 0.1 min observed for ENR ($n=20$).

Mean recoveries from spiked hair samples ranged between 62.4 and 77.1% for ENR and between 62.6 and 76.0% for CPR. The limits of detection (LOD; $s/n=3$) and the limits of quantitation (LOQ; $s/n=10$), calculated taking into account the chromatographic LOD, the dilution factor (5) and the overall recovery (Table 1), were at the ng g^{-1} level, which is very useful for a screening method.

Reproducibility was evaluated by analyzing contaminated hair samples ($n=5$) in triplicate. The mean RSD for the whole analysis was always better than 11% for both ENR and CPR. Recoveries for spiked edible liver ($n=3$) and muscle ($n=3$) tissues ranged from 62.0 to 87.1% for ENR and 42.9 to 46.9% for CPR. LODs varied between 2 and 3 ng g^{-1} for ENR and between 5 and 6 ng g^{-1} for CPR.

Accumulation of quinolones in animal hair and edible tissues

Hair samples taken just before the pharmacological treatments (WP1, NoWP1) were started, as well as samples obtained from control animals, gave negative results for both CPR and ENR (concentration $< \text{LOD}$), whereas residual concentrations of ENR and CPR were found in all hair samples obtained after the pharmacological treatments had been started (Table 2). These values are in good agreement with those reported by Dunnett et al., [18] in his study of racehorse hair.

Table 2 Residual quinolone levels found in hair, liver and muscle from treated animals

| Animal | Treatment | Sampling | Hair samples | | | | | | | | Edible tissues | | | |
|--------|-----------|----------|--------------------|-------|--------------------|-------|--------------------|------|--------------------|-------|---|------|--------------------|-----|
| | | | ENR | | | | CPR | | | | Marker residue concentration ^a | | | |
| | | | Pigmented | | Unpigmented | | Pigmented | | Unpigmented | | Liver | | Muscle | |
| | | | ng g^{-1} | SD | ng g^{-1} | SD | ng g^{-1} | SD | ng g^{-1} | SD | ng g^{-1} | SD | ng g^{-1} | SD |
| Cattle | NoWP | NoWP1 | $< \text{LOD}$ | | $< \text{LOD}$ | | $< \text{LOD}$ | | $< \text{LOD}$ | | | | | |
| | | NoWP2 | 52.0 | 15.9 | 96.9 | 24.5 | 31.6 | 8.7 | 26.6 | 6.2 | 173.3 | 35.0 | 35.8 | 9.4 |
| | | NG-3 | 32.6 | 6.2 | 45.6 | 13.0 | 34.5 | 7.1 | 43.3 | 10.2 | | | | |
| | WP | WP1 | $< \text{LOD}$ | | $< \text{LOD}$ | | $< \text{LOD}$ | | $< \text{LOD}$ | | | | | |
| | | WP2 | 603.3 | 182.0 | 32.1 | 9.0 | 88.9 | 25.5 | 28.8 | 9.2 | | | | |
| | | WP3 | 1707.0 | 297.1 | 33.5 | 7.0 | 187.5 | 27.7 | 22.9 | 7.2 | $< \text{LOD}$ | | $< \text{LOD}$ | |
| | | NG-1 | 1368.5 | 581.6 | 35.2 | 8.2 | 148.8 | 54.1 | 20.2 | 2.5 | | | | |
| Pigs | NoWP | NG-2 | 2517.7 | 264.1 | 22.6 | 4.4 | 254.4 | 57.1 | 20.1 | 0.4 | | | | |
| | | NoWP1 | na | na | $< \text{LOD}$ | | na | na | $< \text{LOD}$ | | | | | |
| | | NoWP2 | na | na | 1140.4 | 734.8 | na | na | 152.3 | 140.5 | 36.9 | 13.9 | 19.2 | 3.0 |

Each value is the mean of $n=2$ animals and $n=2$ independent analyses

^a Sum of ENR + CPR as required in [4, 6].

n.a., not available

ENR and CPR residues were detectable in cattle hair seven days after the beginning of the treatment (NoWP2), and they were still detectable up to five weeks after (WP3). Very low (near the LOQ) ENR concentrations were found in pigmented cattle hair samples taken immediately after the pharmacological treatment, but the accumulation increased significantly with time, up to 1,707 ng g⁻¹ in WP3 samples. The evolution of CPR concentration in pigmented hair was very similar. Unpigmented cattle hairs showed more limited ENR accumulation and values of CPR similar to those for the pigmented ones (Table 2).

ANOVA confirmed that pigmentation significantly ($p < 0.01$) affects ENR and CPR accumulation, as has already been observed in human hair [17] and horsehair [18]. Pigmented hair contains much more melanin (a polymer formed from indolequinone and dihydroxyindole carboxylic acid, which has a great affinity for basic compounds) than unpigmented ones. The selective accumulation of the mother drug ENR in pigmented hairs, which also influences the ratio ENR/CPR, could be due to a rapid and strong interaction between ENR and melanin [18] in the hair bulb when the serum levels of the mother drug are still high.

The progressive increase in ENR and CPR contents in pigmented hair samples after drug administration, as well as the high concentrations of ENR and CPR in new hair shafts grown several days after the end of treatment (NG-1, NG-2), provide further evidence that the prevalent mechanism for the accumulation of these compounds in calf and pig hair is the endogenous pathway [20].

High residual concentrations of ENR and CPR were also detected in pig hair samples (Table 2). Pigs received fivefold more ENR than calves, but ENR accumulated to levels 30–50-fold greater in pig hair than in the corresponding unpigmented calf hairs. Even if a direct comparison is not appropriate for our experimental conditions, it seems that both ENR and CPR accumulated more easily in pig hair than in the corresponding unpigmented calf hairs. Further specific studies could explain whether this behavior is a consequence of metabolic differences between species or whether it depends on the kind of administration used.

Detectable residues of ENR and CPR (Table 2) were found in edible tissues from animals treated without a withdrawal period (NoWP2). In all cases, the concentrations of marker residue (as sum of ENR + CPR) in edible tissues were always low, varying from 19.2 to 173.3 ng g⁻¹ (Table 2), due to the rapid elimination of quinolone residues in edible tissues [6]. This well-known behavior of quinolones [25, 26] makes it difficult to detect the illegal use of these drugs through the analysis of conventional samples.

Conclusions

A simplified HPLC method with fluorimetric detection was developed to detect trace residues of both ENR and CPR in pig and calf hairs. Hair samples (100 mg) were extracted with 4 mL of acidified (TFA 0.2 M) methanol for 24 h at 70 °C. The treatment of the extract, which includes solvent evaporation but does not require any further purification steps, allows the detection of ENR and CPR residues in hair samples with LOQs (between 12–16 ng g⁻¹) that are better than those obtained in horsehair by HPLC-DAD [18].

The accumulation of both ENR and CPR was observed for the first time in pig and calf hairs after therapeutic treatment with ENR. Even though these results should be confirmed on larger populations, hair was demonstrated to accumulate ENR and CPR more efficiently and with a larger time window for detection than in liver and muscle, which are generally used as the target samples in residue monitoring plans. Similar results were obtained in previous studies performed with sulphamethazine [27]. These data corroborate the suitability of hair analysis for monitoring the administration of regulated veterinary drugs to food-producing animals.

Even though the presence of residues in hair does not represent a food safety issue, the detection of quinolone residues in hair could be a valid tool for assessing the retrospective administration of illegal doses of ENR to livestock animals and for improving the efficacy of screening procedures used in official control plans.

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