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Investigation into the experimental protocols required to determine maximum residue limits (MRLs) in honey†

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There is current debate within the EU, and internationally, on how withdrawal periods and maximum residue limits (MRLs) may be set for honey production. Whilst comprehensive EU guidelines exist for calculating the withdrawal times of veterinary medicines in most food-producing species, the analytical variables to be studied for bees/honey are not well defined. The objective of this study was therefore to investigate and understand the factors, for example sampling variability, that is important in the development of a harmonized protocol that can be used to generate the robust scientific data necessary to assist risk assessors in proposing MRLs for honey.

Ten bee colonies were treated in the spring with a model compound (ciprofloxacin). One hive was used to study intra-hive variation in residue concentrations and the other nine were used in an inter-hive study over a 41-week sampling period. All samples were analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The highest mean concentration from nine hives used in the inter-hive study was 4627 μg/kg eight days (D8) after treatment. The concentration of ciprofloxacin declined to an average concentration of 1756 μg/kg at D30 and 733 μg/kg at D283 (over-winter sample). A generalized additive model was used to fit a smooth curve for trend estimation. For some individual hives the concentration of ciprofloxacin increased slightly at the later sampling time-points. Consequently it was not possible to interpolate, with confidence, a finite withdrawal period for ciprofloxacin at theoretical MRLs between 25 and 500 μg/kg. The observed variation in concentration of ciprofloxacin between hives indicates that the validity of the EU guideline for bees/honey, which requires five samples from five hives to calculate a withdrawal period, may require revision. © 2012 Crown copyright. Drug Testing and Analysis © 2012 John Wiley & Sons, Ltd.

Keywords: ciprofloxacin; honey; veterinary drug residues; apiculture; sampling statistics

Introduction

Since pollination by bees is essential for the sustainability of many sectors of agricultural production, maintaining the health of bee colonies is of great economic importance. Bee colonies are susceptible to a number of infestations and diseases including Varroa and Foulbrood[1] but, worldwide, there are relatively few drugs legally permitted for control.[2] Although the United States of America has authorized the use of oxytetracycline and tylosin to treat American Foulbrood (AFB),[3] the EU has yet to authorize antimicrobial-based veterinary medicines for the treatment of bees.[4] There is current debate within the EU, and internationally, on how maximum residue limits (MRLs) and withdrawal periods may be set for honey production. Whilst comprehensive guidelines exist for calculating the withdrawal time of veterinary medicines in most food producing species the analytical variables are not that well defined for bees/honey. For example, the *EU Notice to Applicants and Guideline for the Establishment of MRLs for Residues of Veterinary Medicinal Products in Foodstuffs of Animal Origin* (Volume 8)[5] states: 'Where relevant for the proposals for MRLs the expert should present and discuss a summary table of approximate withdrawal periods for each species of food-producing animal as well as their edible products, such as milk, eggs and honey, which could be realistically observed under conditions of good practice in the use of veterinary medicinal products.'

The withdrawal periods for animal slaughter as well as for the production of milk, eggs, and honey for human consumption are determined from the results of suitable residue depletion studies using the formulation intended for marketing. For honey, this depletion study should comprise "5 samples from each of 5 hives, the time points to consider should be defined according to the period of treatment and the production of honey; the withdrawal period should provide a high degree of assurance both to the producers and the consumers that the concentrations of residues in foods derived from treated animals are not above the permitted concentrations."[5]

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Whilst these guidelines are helpful, previous studies have demonstrated that veterinary medicines can persist in honey collected from hives several months after treatment.[6–12] Furthermore, the distribution of residues within and between hives is often variable. Further research is therefore needed to determine the most important factors that should be considered when withdrawal periods are to be calculated for honey.

The objective of this research project was to use the fluoroquinolone antibiotic ciprofloxacin as a model compound to assess experimental variables including (1) the number of hives, (2) sampling frequency (within and between super boxes), and (3) sampling location within the hives. Data from these experiments would provide statistical information that could be used to define the experimental requirements for a suitable residue depletion study and hence assist with the calculation of fit for purpose withdrawal periods.

Methods and materials

Bee colonies and treatments

The dosing study was carried out in May 2009 just prior to the main honey flow. Standardized free flying colonies of UK honeybees (*Apis mellifera* L.), housed in double Smith brood chambers (box in which the queen is confined and brood is reared) with 11 British standard frames (33.6 cm by 20.4 cm giving 685.4 cm^2 per side of brood frame) per brood box and at least one super box (a shallower box in which the bees store honey and which is placed above a queen excluder and the brood chambers), were used in this study. The super box is also referred to as the honey super. The colonies were owned and maintained by the Food and Environment Research Agency (Fera), National Bee Unit. At the start of the study these colonies showed no clinical signs of European or American Foulbrood, Sacbrood, or Baldbrood and had only a low incidence of Chalkbrood. Ten colonies were treated with ciprofloxacin and two control colonies (not treated) were located at experimental apiaries separated by a distance of 10 km, to reduce the risk of cross-contamination.

The ten treated colonies were dosed with a solution of 1 g of ciprofloxacin in 120 ml aqueous sucrose solution (50 *w/v*). The treatment comb was placed in the top brood box, two frames from the edge of the brood nest with the treated side of the frame out. The two control colonies were fed with untreated sucrose using the same method of application. After sampling in October 2010, the colonies were fed with 50% *w/v* sucrose using a rapid tray feeder. All bee husbandry was carried out using typical beekeeping practices. Super boxes were added using the 'top-supering method' where a new empty super box is placed directly on the top of the super box(es).

Sampling

The sampling time points used in this study are summarized in Table 1. In May 2009, two to four days before treatment (−D2 to -D4) samples of up to 100 g of honey were taken from each colony to confirm that antibiotic residues were not present. Samples of super honey were collected at D3 after treatment and at approximately weekly intervals during the bee-keeping season. Additional samples were collected at D132 and post-wintering samples were collected at D283 in February 2010.

Nine of the hives were designated for an assessment of inter-hive variability and one hive designated for an assessment of intra-hive variation. The number of samples collected was different in each case.

For the assessment of inter-hive variability two comb samples (approximately 8 cm by 10 cm) were taken from random locations in each super box of each hive per time point. The two samples were combined before filtering. For the assessment of intra-hive variability two samples were collected, at each time point, from each of the ten frames in each super-box. The sampling positions were selected randomly at the time of collection. These samples were filtered and analysed as individual samples.

All honey samples were extracted by filtering through cloth (mesh size approx. 0.25 mm x 0.25 mm) into a clean container and stored at −20°C prior to analysis.

Table 1. Summary of results for ciprofloxacin in super honey (intra-hive distribution study)

		Super box 1				Super box 2				Super box 3 *			
Sampling time point	Days from dosing	Concentration (μg/kg)			n	Concentration (μg/kg)			n	Concentration (μg/kg)			n
		mean	Min.	Max.		mean	Min.	Max.		mean	Min.	Max.	
1	3	3849	775	7667	19	4254	472	5804	10	n/a	n/a	n/a	n/a
2	8	3187	1856	5044	19	2891	1858	4770	14	n/a	n/a	n/a	n/a
3	16	1422	404	3073	19	1274	204	4007	18	n/a	n/a	n/a	n/a
4	22	804	79	1878	20	863	148	3819	20	n/a	n/a	n/a	n/a
5	30	691	204	1843	20	753	287	2431	19	291	246	321	12
6	37	735	186	1584	20	634	225	2243	20	301	161	375	16
7	43	376	77	1010	20	583	234	1742	20	222	86	340	18
8	51	363	47	864	20	362	138	2142	19	175	90	340	18
9	59	300	78	945	20	277	76	906	18	216	115	315	18
10	66	232	109	772	20	327	114	1325	17	154	101	198	18
11	73	223	103	457	11	448	84	1491	16	187	102	288	18
12	132	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	214	99	365	14

n/a No honey available for sampling.
* Super box 3 was only added after time point 4.

Apparatus and reagents

Ciprofloxacin was purchased from Sigma Aldrich (Dorset, UK) and ciprofloxacin - d8 from Witega Laboratorien (Berlin, Germany). OASIS MCX solid-phase extraction (SPE) cartridges (3 ml/60 mg) were purchased from Waters (Manchester, UK). All other reagents were of analytical grade and obtained from either BDH (Poole, UK) or Fisher Scientific (Loughborough, UK).

Preparation of standard solutions

A stock standard of ciprofloxacin (500 μg /ml) was prepared in acidified methanol with a shelf life of six months, when kept at -20 °C.

Extraction procedures

Extraction of super honey (control samples and samples containing residues at ≤ 100 μg/kg)

Honey (1g) was dissolved in ammonium acetate (0.005 M, pH4, 10 ml) and ciprofloxacin -d8 added as an internal standard. After heating at 40°C (in a water bath) for 10 minutes the extract was allowed to cool to room temperature (20°C) and then loaded onto an OASIS MCX SPE cartridge (pre-conditioned with methanol (2 ml), HPLC grade water (2 ml) and extraction buffer (2 ml)). The SPE cartridge was washed with HPLC grade water and methanol (2 ml of each) before elution with ammonia solution (25%, *v/v*) in acetonitrile (3 ml). The eluate was evaporated to dryness at 55°C under a stream of nitrogen and reconstituted in formic acid 0.1 % *v/v* (1 ml). Extracts were analyzed directly by LC-MS/MS using matrix-matched calibration standards over the range 5–200 μg/kg. Matrix-matched standards were prepared by adding the appropriate amount of analyte to blank matrix at the final reconstitution stage (after extraction and clean-up).

Dilution step for honey samples containing residues at > 100 ≤ 1,000 μg/kg. Extracts from above were diluted 10-fold and matrix-matched calibration standards were prepared over the range 50 to 2000 μg/kg.

Dilution of samples containing residues at > 1000 μg/kg. Honey (1 g) was dissolved with formic acid (0.1 %, 1,000 ml) and ciprofloxacin- d8 added. The diluted samples were analyzed directly by LC-MS/MS using matrix-matched calibration standards over the range 500–15,000 μg/kg.

Quantification. The LC-MS/MS system comprised a Quattro Ultima Triple Quadrupole (Waters, Manchester, UK) coupled to an Alliance 2695 Separations Module (Waters) controlled by Mass Lynx version 4.1. An isocratic separation was performed using a Hypurity C_{18} (100 mm x 2.1 mm, particle size 3 μm) HPLC column (ThermoFisher Scientific, San Jose, CA, USA), installed with a filter. Mobile phase was 0.1% (*v/v*) formic acid in water/methanol/acetonitrile, 80/10/10 *v/v/v* with a flow rate of 0.3 ml/min and an injection volume of 40 μl. The LC-MS/MS was operated in positive electrospray ionization mode. The capillary voltage was 3.0 kV, the cone voltage 35V, the source temperature 120 °C and the desolvation gas temperature 350 °C at a flow rate of 1.3×10^4 ml min^{-1} (N_2). The transitions monitored were *m/z* 332.1 > 288.1 at collision energy of 15eV and *m/z* 332.1 > 245.1 at collision energy of 25eV for ciprofloxacin, and *m/z* 340.1 > 296.1 at a collision energy of 15 eV for d8-ciprofloxacin. The limit of quantification (equivalent to the concentration of the lowest matrix-matched calibration standard) for ciprofloxacin was 5 μg/kg.

Method validation and routine quality control

Initial validation of the methods involved spiking blank honey samples with ciprofloxacin at concentrations of 100 and 250 μg/kg (SPE method), 1,000 μg/kg (SPE method with dilution), and 10,000 μg/kg ('dilute and shoot'). Honey samples spiked with ciprofloxacin at concentrations between 25 and 10,000 μg/kg were also included in each batch of samples as routine quality control. The numbers of replicate measurements conducted at each concentration are given in Table 2.

Table 2. Summary of routine quality control data for ciprofloxacin spiked into honey

Concentration (μg/kg)	Apparent recovery (%)	RSD (%)	n
25	112	n/a	2
50	108	n/a	1
100	111	8	21
250	106	6	9
500	109	11	3
1,000	107	9	10
2500	100	5	12
10,000	99	6	22

Stability study of ciprofloxacin in honey and solvent

Aliquots (1g) of blank 'raw' honey (filtered only after collection) were spiked with ciprofloxacin at 100 μg/kg. These samples were stored in the dark for 90 days under four different temperatures; 38°C (approximate core temperature in a bee hive), room temperature, -20°C and at -80°C. Aqueous solutions of ciprofloxacin (0.5 μg/ml in water) were also analyzed over a 6-month period (stored at 4°C in the dark) to monitor solvent stability. For each storage condition, two replicate aliquots were withdrawn at intervals and quantified using the LC-MS/MS method.

Statistical analysis

Calculation of withdrawal times

A typical method to model a decrease in concentration of a chemical over time is to use an exponential decay curve. However, as the observed residue concentration increased at later time points this model was not appropriate. The observed pattern did not suggest any known parametric functional form although the obvious candidates were explored. Consequently, an arbitrary smooth interpolating function, known as a generalized additive model (GAM),[13] was used to allow estimation by interpolation of concentration between observed time points; this has the additional advantage of flexibility to adapt to other patterns of concentration over time in the future. All calculations were performed in R version 2.10.1 (www.r-project.org). Five degrees of freedom were specified as the maximum number for the GAM function used for all the models to allow sufficient flexibility to capture the shape of the observed response, while smoothing out small-scale fluctuations.

Assessment of intra-hive variation in residue concentrations

The model for the trend in concentration over time did not account for all the variation in the data. Consequently, the source (s) of the remaining variation was further studied by statistical

analysis after accounting for the trend. A series of models were used to evaluate the intra-hive residue data. These included:

Model 1: $\log(\text{Concentration})_{\text{Time}} = s(\text{Time}) + \varepsilon$
Model 2: $\log(\text{Concentration})_{\text{Time, SB}} = s(\text{Time}) + \text{Super Box}_{\text{SB}} + \varepsilon$
Model 3: $\text{Residuals} = c_3 + \varepsilon_{\text{Frame}} + \varepsilon$

where

s = smoothing function (using the GAM function in the software package)
c = an estimated constant; subscript identifies model
ε = error term (normally distributed with mean = 0 and variance = σ^2)

Models may have more than one error term:
ε without subscript refers to the individual data items, ε with subscript represents the variation in means for the subscripted item
Super Box $_{SB}$ = the fixed effect for SB (i.e. difference from general mean for each super box)
Residuals = residuals after fitting GAM (i.e. eliminating the trend over time); the models were fitted sequentially because it is not possible to use a GAM directly in a mixed model.

Assessment of inter-hive variation in residue concentrations

Models 1, 2 and two further models (symbols as above) were used to assess the inter-hive data after accounting for the trend over time:

Model 4: $\text{Residuals} = c_4 + \varepsilon_{\text{Hive}} + \varepsilon$
Model 5: $\text{Residuals} = c_5 + \varepsilon_{\text{Hive}} + \varepsilon_{\text{SB|Hive}} + \varepsilon$
$\varepsilon_{\text{SB|Hive}}$ = the variation in super boxes within a given hive

Calculation of minimum sample numbers (inter-hive study)

In this assessment, the objective was to calculate the minimum number of samples required to achieve an acceptable relative standard uncertainty under realistic field conditions, hence the inter-hive data were used. For similar analytical methods used for pesticide residue measurement, an acceptable level of uncertainty is taken to be 0.25[14] with the total relative standard uncertainty (RSU_{Total}) represented as follows:

$$RSU_{Total} = \sqrt{RSD^2_{analysis} + RSD^2_{sampling}} \quad (1)$$

RSU_{Total} is an estimate of the relative standard uncertainty associated with a concentration estimate. It includes the effects of both variation between and within hives and the uncertainty associated with the method used to measure concentrations. Mathematically it can be treated like a relative standard deviation. The relative standard deviation for the analytical method ($RSD_{analysis}$) was calculated at different concentrations using validation data.

The $RSD_{sampling}$ can be expressed for the inter-hive study as:

$$RSD_{Sampling} = \sqrt{\frac{RSD^2_{between\ hives}}{n_{Hive}} + \frac{RSD^2_{between\ super\ boxes}}{n_{Hive}n_{Super\ box}} + \frac{RSD^2_{between\ replicates}}{n_{Hive}n_{Super\ box}n_{replicates}}} \quad (2)$$

To calculate the RSD for each of the sampling components the formula was used:

$$RSD_x = \sqrt{e^{s_x^2} - 1} \quad (3)$$

where s_x^2 is the estimated variance component for x.

Results and discussion

Method validation and quality control

The measured concentrations of ciprofloxacin in spiked extracts (method validation and analytical quality control combined) were within 99–112% of the spiked concentrations with associated RSD of ≤ 9 % (n = 80) as summarized in Table 2. These results confirmed that the method was fit-for-purpose and under control throughout the study.

Ciprofloxacin spiked into unprocessed blank honey was stable during storage for 90 days in the dark under all conditions; at +38°C, room temperature, -20°C and at −80°C. It can therefore be concluded that the observed decline in ciprofloxacin concentration in the super honey in the hive is not a function of chemical degradation. Ciprofloxacin standard at a concentration of 0.5 μg/ml prepared in water was also stable after 188 days at 4°C in the dark.

Intra-hive distribution study

One of the ten hives was selected at random for the intra-hive distribution study. The weather conditions throughout the duration of the experiment were favourable and consequently the honey flow was relatively high (40–50 kg honey per hive). Two super boxes were in place at the start of the honey flow and a third super-box was added at time point four (D22). For each time point, the sampling plan detailed the collection of 20 samples per hive (two samples from ten frames) but honey was not always available for collection from each frame. Thus, the actual numbers of samples collected from each hive varied by time point; the actual number of samples collected is listed in Table 1. A summary of the mean, minimum and maximum concentrations of ciprofloxacin at each time point are also presented in Table 1. These data are very similar to the mean concentrations of ciprofloxacin observed in the other nine hives used in this study (Table 3).

In the intra-hive study, the concentration of ciprofloxacin was observed to increase slightly at some of the later time points (e.g. 448 μg/kg was found in super box 2 at Day 73, compared to 327 μg/kg at Day 66). The increase in residue concentration at later time points means that a statistical model based on a standard exponential decay curve could not be used. Statistical analysis of the results was therefore conducted using Models 1, 2 and 3. These results from fitting Model 3 showed that the variation in concentration of ciprofloxacin within a frame is greater than variation between frames, at least when taking small (approximately 10–20 g) samples. The variance component for between frames was 0.0019 whilst the within frame variation was 0.3973 with the GAM (Model 2) accounting for 96.2% of the total variation.

Table 3. Summary of results for ciprofloxacin in super honey (inter-hive study)

Sampling time point	Days from treatment	Concentration (μg kg/kg)†			n*
		Mean	Min.	Max.	
1	3	4584	571	11796	17
2	8	4627	1272	9908	17
3	16	3403	717	9704	17
4	22	2308	461	5948	17
5	30	1756	452	5987	21
6	37	1697	295	5211	21
7	43	1685	299	5583	21
8	51	1211	213	5226	21
9	59	1160	248	4912	21
10	66	1012	256	4543	21
11	73	1322	305	7712	21
12	132	1074	409	2257	17
13	283	733	91	2054	17

* Equals number of super boxes analyzed.

† Mean concentration based on the overall average of two replicate analyses per super box.

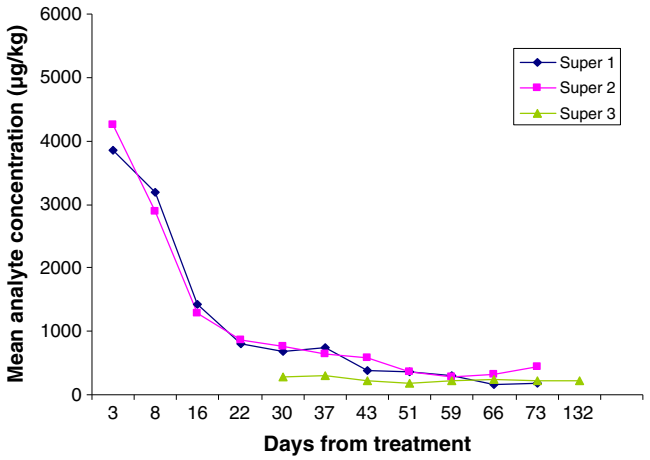

Figure 1. Intra-hive distribution study; ciprofloxacin depletion over time.

The depletion trend was similar for all 3 super boxes (Figure 1) but the mean concentrations were significantly different ($p < 0.001$) between super box 1 and super box 3. Although bees can move honey within and between super boxes it is unlikely that the honey is completely mixed during periods of high honey flow. These results are possibly explained if the honey (containing residues of ciprofloxacin), from super boxes 1 and 2, is diluted with freshly produced honey in super box 3.

In theory a predicted withdrawal time can be interpolated using the GAM model approach but only if the MRL was set at high concentrations. In practice, a finite withdrawal time cannot be predicted at realistic MRL concentrations (e.g. 100 μg/kg) because of the slight increase in drug concentration observed at the later time points.

Collecting such a large number of samples (20 per super box, per time point) proved to be impractical as it caused too much disturbance to the bees. Therefore, results from the inter-hive study, which was based on two samples combined (total approx. 100 g) collected from each super-box at each time point from a greater number of hives is more practical, and more representative of the super honey produced in an apiary.

Inter-hive study

The results obtained for ciprofloxacin at Day 0 (control samples) were all <5 μg/kg.

The mean concentrations of ciprofloxacin measured in the super honey collected from treated colonies are shown in Table 3. The highest mean concentration for the nine hives was 4627 μg/kg at sampling point 2, eight days after treatment. The concentration of ciprofloxacin declined to an average concentration of 1756 μg/kg at Day 30 and 733 μg/kg at Day 283 (over-winter sample). The highest concentration found in an individual hive was 11796 μg/kg at Day 3.

The depletion profile of ciprofloxacin in honey (Figure 2) is very similar to the depletion profiles reported for oxytetracycline,[6] tylosin,[7] chloramphenicol,[8] and lincomycin[9] and the detection of residues of ciprofloxacin in overwintered samples (Day 283) demonstrates that parent ciprofloxacin is a suitable marker to detect the presence of ciprofloxacin in apiculture. The depletion is not due to chemical degradation but dilution of ciprofloxacin by the increase in honey mass during the honey flow.

As with the intra-hive distribution study, the concentration of ciprofloxacin in the inter-hive study was observed to increase slightly or stabilize at later time points. Consequently a standard exponential decay curve would be inappropriate. GAM models were therefore used.

Models 1, 2, 4, and 5 could not be used to predict a withdrawal time for a number of arbitrary MRLs between 25 and 500 μg/kg, because of the slight increase in concentration of ciprofloxacin at later time points. This suggests that a regression model approach may not be appropriate for calculating withdrawal times. An example to illustrate the prediction and 95% prediction intervals using Model 1 are given in Figure 3. These predictions for the simplest model (where the data is smoothed over time) clearly demonstrate the challenge of dealing with data with a high degree of variability, typical of a dynamic biological system.

Fitting Model 2 indicated that there are significant differences ($p < 0.001$) between super boxes 1 and 3. By fitting Model 4 to the residuals (after fitting GAM Model 2) the variance component was calculated to be 0.24 for inter-hive variation and 0.29 for intra-hive variation indicating that there is more variation within each hive (between super boxes) than between hives. Still, as

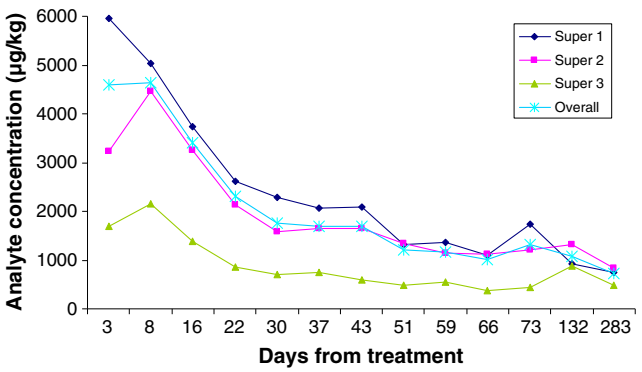

Figure 2. Inter-hive distribution study; ciprofloxacin depletion over time.

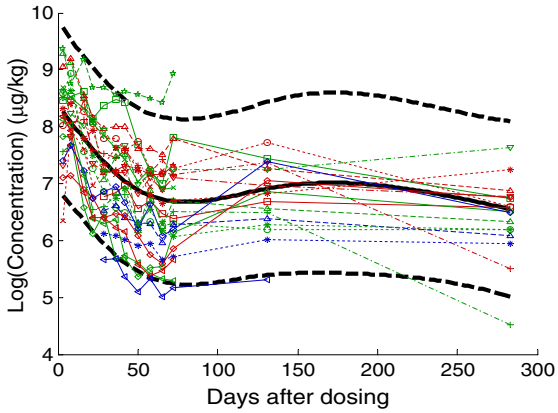

Figure 3. Prediction and 95% prediction intervals for log (concentration) of ciprofloxacin over time from the inter-hive data when Model 1 is fitted to the means of the super boxes. The black solid line is the prediction and the thick dashed lines the 95% prediction intervals. The dashed lines indicate super box 1, the dotted lines indicate super box 2 and the dash-dot lines indicate super box 3.

the inter-hive variation is substantial (GAM Model 2 only accounted for 45% of the variation) it is not possible to interpolate these data with confidence.

Calculation of the variance components with Model 1 and Model 5 fitted sequentially to replicates, with both hives and super boxes considered as random effects, resulted in the same conclusions. The largest variation is between super boxes (0.2617), then between hives (0.2121), and the smallest variation is between samples (0.1555).

It was concluded that using any of the GAM models to predict a withdrawal time for theoretical MRLs between 25 and 500 µg/ kg is extremely difficult.

The large variation, not explained by the GAM models (e.g. Model 1 only accounts for 36.3% of the variation), suggests that a lot of variation is due to factors not included in the model. For example, the different concentrations of ciprofloxacin in super boxes over time in different hives suggest that there is variation between the behaviour of bees in different hives; some are mixing honey between super boxes more efficiently than others. Since it is unlikely that these behaviours could be controlled it will be necessary to modify the experimental design if a greater proportion of the variation is to be accounted for in future studies.

The data from this current study were therefore used to calculate the number of hives and samples that might be required to reduce the variation, due to sampling, to an acceptable level.

Calculation of minimum sample numbers (inter-hive study)

In the absence of an accepted target uncertainty for bees/honey the uncertainty for the measurement of pesticide residues, 0.25, recommended by Codex was employed for this evaluation.[14]

First, the relative standard deviations for the analysis (RSD_A) were calculated from the analytical quality control data (several analytical batches). The relative standard deviations (RSD_A) were less than the equivalent Horwitz relative standard deviations (RSD_H). In fact the Horrat ratio (the ratio of these relative standard deviations, RSD_A/RSD_H) was <0.5 in most cases. Hence the expected uncertainty associated with analytical results produced by this method, is estimated using modified Horwitz relative standard deviation (RSD_H).[15] Given that it is unlikely that the uncertainty associated with the analytical method can be reduced further, the sampling uncertainty needs to be as small as possible with respect to the uncertainty from the analytical method. (Table 4)

Table 4. Coefficients and t tests for super box effects for log (Concentration) of ciprofloxacin in inter-hive study

Fixed term	Std. Error	t value	Pr($>$ |t|)
Super box 1	0.064	112.355	$<$ 0.001
Super box 2	0.092	0.056	0.955
Super box 3	0.132	-6.226	$<$ 0.001

The RSU_{Total} was calculated for different sampling schemes (numbers of replicates, hives, super boxes) at 'arbitrary MRL' concentrations using a target uncertainty value of 0.25[14] as a guideline. The relative numbers of hives and samples required to meet the target uncertainty, at an arbitrary MRL of 100 µg/kg, are presented in Table 5. These data show that, if five samples were collected from five hives (the current EU recommended practice) an RSU_{Total} of 0.37 would have resulted. A minimum number of 40 hives, with two super boxes (and at least two samples per hive), is required to meet a 0.25 target uncertainty. Clearly, these data show that the number of hives sampled is more influential than the number of samples. However, it is important to note that collecting larger sample sizes from individual hives is likely to reduce the between-sample variability and may enable the number of hives to be reduced below 40.

Table 5. The variation in Relative Standard Uncertainty (RSU_{total}) depending upon the relative number of hives and samples per super box

	Number of samples to be taken per super box per time point		
n	2	5	10
	Relative Standard Uncertainty (RSU_{total})		
5	0.37	0.37	0.36
10	0.31	0.31	0.30
20	0.27	0.27	0.27
30	0.26	0.26	0.26
40	0.25	0.25	0.25
50	0.25	0.25	0.25

n = number of hives to be sampled, each with two super boxes.

Also, the target uncertainty (0.25) used in this illustration may not be appropriate. It would be helpful if an official guideline value was available to assist with calculating the number of hives required to determine a robust withdrawal time.

Conclusions

Because of the persistence (283 days after treatment), the large inter-hive variability of residues of ciprofloxacin, and the fact that residue concentrations increased in some hives at the end of the study it was not possible to calculate a robust withdrawal period for ciprofloxacin at theoretical MRLs between 25 and 500 µg/kg.

However, for a veterinary medicine with less persistent residues it may be possible to calculate a robust withdrawal time.

The fact that ciprofloxacin was detected 283 days after treatment indicates that the parent compound is a suitable marker for the detection of use or abuse of ciprofloxacin in apiculture.

The observed large inter-hive variation in concentrations of residues of ciprofloxacin, not unexpected in a biological system, indicates that the validity of the current EU guidelines for bees/honey, which requires five samples from five hives to calculate a withdrawal period, may require revision. It would be helpful if the guidelines also recommended an acceptable total relative uncertainty (RSU_{Total}). The results of this study indicate that increasing the number of hives is more influential than the number of samples per hive on decreasing the relative standard uncertainty (RSU_{Total}).

Although the results of the current study provide valuable information this investigation was undertaken using one model compound during one season at one geographical location. Ideally, further studies should be conducted to assess other potential variables such as the effects of the use of other model compounds with different application regimes, different geographic locations, different climatic/seasonal variables and different bee husbandry practices.

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Conflicts of interest

The authors have no conflicts of interest to declare.

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