Quantitation of neonicotinoid insecticide residues in experimentally poisoned honey bees

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Abstract

Background: In cases of poisoning incidents, the standard procedures consider the sampling of dead honeybees to define the active ingredients (a.i.) involved and to determine if the quantity of the residue present can be considered responsible of the death. However one should verify the loss of residues in the period from the discovery of the dead honeybees to their analysis. Since honey bee mortalities due to neonicotinoid insecticides have been recently reported, thiametoxam, clothianidin, imidacloprid residues were determined in experimentally poisoned honey bees.

Results: Oral and indirect contact trials were carried out for each pesticide, using commercial formulations. Honey bees that died during the trials were stored at -18 °C and analyzed through a LC-MS/MS analytical procedure adapted from AOAC methods. The quantity of insecticide residues that were detected resulted much lower than the administered residues. Honey bees that did not die within six hours from the trial start were also analyzed and the quantities of insecticide residues resulted much lower than those found in the dead honey bees.

Conclusion: On the basis of these results, the determination of the Subsequent Residue Level or of a similar index should be required during the normal procedures of authorization for the use of pesticides.

Keywords: poisoning incidents, clothianidin, imidacloprid, thiametoxam

Introduction

The threat posed to honey bees (*Apis mellifera* L.) and other pollinators by most insecticides and some fungicides and herbicides are generally recognized;[1,2] therefore risk assessment schemes had been developed and are currently implemented so as to reduce honey bee losses and inform correctly the users on possible side effects of pesticides.[3,4] Nevertheless unexpected contamination routes, like the harvesting of microencapsulated insecticides by pollen foragers[5,6] or, more recently, the release of dusts from insecticide coated seed when drilled with inadequate machinery,[7-12] may arise; moreover, the incorrect or unauthorised use of pesticides may also result in severe honey bee losses.

In the last decades, different approaches for detecting and monitoring such incidents were devised.[13-16] In any case, the sampling of dead honeybees to single out the active ingredients (a.i.) involved is a crucial point of any procedure, especially when the amount of residue detected is taken into account to determine if the a.i. can be considered responsible for honey bee death. However one should also consider the loss of residues in the period from the discovery of the dead honeybees and their analysis. In former times the determination of the Subsequent Residue Level (SRL) was proposed to quantify to a certain extent these losses,[17] but a somehow more sensitive method could be advisable.

In the recent years, both pollinator decline and colony collapse disorder (CCD) have become serious concerns that could ultimately impair the production of many crops in Europe and in the United States.[18-22] Pesticide (neonicotinoids in particular) use has been identified as a potential contributing factor to CCD and may be one of the environmental stressors contributing to pollinator declines, along with other factors such as new and re-emerging pathogens, habitat loss, pests, and nutritional stress. Most neonicotinoids show a very strong toxicity to pollinating insects and in particular to the honey bee, causing also other effects which are seldom easily identifiable, such as behavioural disturbances, orientation difficulties and impairment of social activities.[2,22-28] Although potential problems could be reduced by appropriate mitigation practices,[1,29] alarming bee mortalities, clearly

due to the use of neonicotinoids either for seed dressing or crop spraying, were recorded in many countries during the past few years, and various limitations in their use were enforced.[9,22]

Therefore, it seemed appropriate to determine clothianidin, imidacloprid and thiametoxam residues in experimentally poisoned honey bees in order to have an estimate of a.i. losses from treatment to sampling and so to gain a better understanding of the role played by these neonicotinoids in poisoning incidents.

Experimental methods

The research was carried out in the laboratory using methods developed at the Di.Va.P.R.A. to test acute oral toxicity (AOT) and indirect contact toxicity (ICT) on honey bees.[30,31] Commercial formulations available in Italy were used: Dantop 50 WG (Isagro Italia s.r.l., Milano, Italy): 50.0% pure clothianidin, hydro dispersible granules; Confidor 200 SL (Bayer S.p.A., Milano, Italy): 17.8% pure imidacloprid, concentrated liquid soluble in water; Actara 25 WG (Syngenta Crop Protection S.p.A., Milano, Italy): 25.0% thiametoxam, hydro dispersible granules. Tests were performed in a dark room at 28-30 °C and 70% relative humidity. Compounds were tested at the highest concentration recommended on the label for crop treatment (field concentration) and they were gradually diluted down to the concentration that caused a mortality not significantly different from that of the untreated controls. Significance was verified with the chi-square test. For each test 40 workers from a single colony were used and the tests were replicated with honey bees from different colonies. Altogether six *A. m. ligustica* colonies from Piedmont (Italy), two *A. m. carnica* colonies from Croatia, and one *A. m. mellifera* colony from France were used. Tested a.i. concentrations and the relative replication number are reported in table 1 for AOT and in table 2 for ICT.

Tab. 1 Amounts of neonicotinoids recovered from dead honey bees after ingestion of decreasing a.i. doses.

Concen-tration (mg L^{-1})	Ingested dose (ID) (ng honey bee^{-1})	Repli-cations (n)	Mortality 24 h mean ± st.dev. (%)	48 h mean ± st.dev. (%)	Detected amount (DA) mean ± st.dev. (ng honey bee^{-1})	DA/ID mean ± st.dev. (%)
clothianidin						
75	2625	1	100.0	100.0	26.60	1.01
7.5	262.5	1	96.7	100.0	5.40	2.06
1.5	52.5	4	98.7 ± 3.0	99.3 ± 1.5	2.52 ± 1.36	4.82 ± 2.60
0.75	26.25	5	94.0 ± 13.4	94.7 ± 11.9	2.36 ± 1.20	9.00 ± 4.58
0.375	13.125	7	91.5 ± 3.8	92.2 ± 3.0	0.80 ± 0.76	6.10 ± 5.79
0.15	5.25	5	70.8 ± 19.2	71.2 ± 18.7	0.94 ± 0.57	17.31 ± 11.07
0.075	2.625	5	53.4 ± 28.7	56.6 ± 26.8	0.45 ± 0.29	16.11 ± 9.26
0.0375	1.3125	3	20.0 ± 13.7	21.7 ± 13.7	0.30 ± 0.01	22.86 ± 0.76
imidacloprid						
150	5250	3	100.0 ± 0.0	100.0 ± 0.0	159.00 ± 1.00	3.03 ± 0.02
3	105	4	45.8 ± 11.1	82.5 ± 13.2	7.73 ± 2.35	7.36 ± 2.24
1.5	52.5	4	43.8 ± 4.5	61.3 ± 20.7	1.97 ± 1.38	3.75 ± 2.63
0.75	26.25	4	42.3 ± 30.6	51.3 ± 30.1	0.20 ± 0.31	0.78 ± 1.18
0.3	10.5	3	26.7 ± 9.5	26.7 ± 9.5	<0.005	<0.047
thiametoxam						
100	3500	2	100.0 ± 0.0	100.0 ± 0.0	11.61 ± 10.46	0.33 ± 0.30
20	700	1	100.0	100.0	3.24	0.46
10	350	2	100.0 ± 0.0	100.0 ± 0.0	3.56 ± 3.73	1.02 ± 1.07
5	175	1	100.0	100.0	2.30	1.31
2	70	3	100.0 ± 0.0	100.0 ± 0.0	0.71 ± 0.59	1.02 ± 0.85
1	35	4	99.5 ± 1.1	99.5 ± 1.1	0.83 ± 0.36	2.37 ± 1.02
0.5	17.5	8	97.2 ± 5.2	97.4 ± 5.3	0.38 ± 0.23	2.19 ± 1.32
0.2	7	5	67.8 ± 8.0	78.1 ± 13.4	0.01 ± 0.01	0.13 ± 0.13
0.1	3.5	7	41.5 ± 37.6	43.9 ± 37.1	0.005 ± 0.002	0.13 ± 0.05
0.05	1.75	4	7.4 ± 8.7	8.5 ± 9.8	<0.005	<0.286

Tab. 2   Amounts of neonicotinoids recovered from dead honey bees as a consequence of indirect contact tests.

| Concentration (mg L^{-1}) | Replications (n) | Mortality | | Detected amount |
		24 h mean ± st.dev. (%)	48 h mean ± st.dev. (%)	mean ± st.dev. (ng honey bee^{-1})
clothianidin				
75	1	100.0	100.0	59.00
37.5	1	100.0	100.0	28.00
15	2	98.3 ± 2.4	100.0 ± 0.0	5.97 ± 0.24
7.5	3	67.3 ± 0.9	76.7 ± 9.4	1.65 ± 0.66
3.75	2	38.8 ± 10.1	51.3 ± 7.5	0.67 ± 0.52
imidacloprid				
150	1	27.5	60.0	21.00
75	1	7.5	32.5	15.60
30	1	20.0	40.0	10.56
15	1	4.3	22.9	0.91
thiametoxam				
100	1	100.0	100.0	27.00
20	2	98.3 ± 2.4	100.0 ± 0.0	2.02 ± 1.15
10	4	91.0 ± 13.0	98.5 ± 1.4	3.36 ± 1.92
5	4	48.6 ± 15.3	64.9 ± 22.8	1.46 ± 0.52
2	2	3.4 ± 3.4	6.7 ± 6.0	0.27 ± 0.01

The ingested dose was calculated from the tested a.i. concentration taking into account that in AOT tests each honey bee ingests 35 μl of sucrose syrup during the allowed one hour feeding period;[30] on the contrary a.i. amounts that penetrate into the honey bees during the ICT tests could not be evaluated.

Tests started at 12.00 h; mortality was checked at 13.00 h (AOT only), 15.00 h, and 18.00 h on the first day of the trial and at 9.00 h, 12.00 h, 15.00 h, and 18.00 h during the following days. The trials lasted three days and mortality percentages at 24 and 48 h were calculated after correction for mortality of the untreated controls.[32]

At every check, dead honey bees were removed from the cages, stored at -18 °C, and shipped frozen to the analytical laboratory for residue determination through a LC-MS/MS analytical procedure adapted from AOAC methods 2007.01 2007;[33] a LC-MSMS apparatus API 3200, AB SCIEX, 110 Marsh Drive, Foster City, California 94404-1121, USA was used. LOD and LOQ were 0.01 μg kg^{-1} and 0.05 μg kg^{-1} respectively. Linear regression of a.i. detected amounts on tested concentrations was calculated with the statistical package PAST;[34] the Ordinary Least Squares algorithm was used and the regression line was forced through zero.

In order to verify if a.i. residues in honey bees that did not die were different from those present in dead ones, special AOT tests were performed. They were started like standard tests, but dead honey bees were removed from the cages six hours since trial start and all surviving honey bees were captured. Dead and alive honey bees were separately frozen at -18 °C and submitted to chemical analysis.

Results

During the trials, the honey bees showed obvious symptoms of poisoning, such as shaking and tremors, uncoordinated and uncontrolled movements, inability to take up a correct position of the body, and prolonged frenetic movements of the legs and rotation when being in the supine position. Direct observation of the behaviour of the honey bees in cages proved that it was transient at a lower concentration. Moreover, the highest concentrations caused extensive vomiting by honey bees, especially in AOT tests.

The amounts of neonicotinoids recovered from honey bees that died in AOT tests are reported in table 1. Replication number is greater for doses causing mortalities between 100 and 50 % because

the resulting variability tends to be higher at these doses. The detected amount/ingested dose ratio is generally rather low and shows great variations between the three a.i. tested and also between doses of the same a.i.; nevertheless regression lines show fair correlations between tested concentrations - and thus ingested doses - and a.i. amounts detected in dead honey bees, while the probability that the two variables were not correlated is extremely low (figure 1).
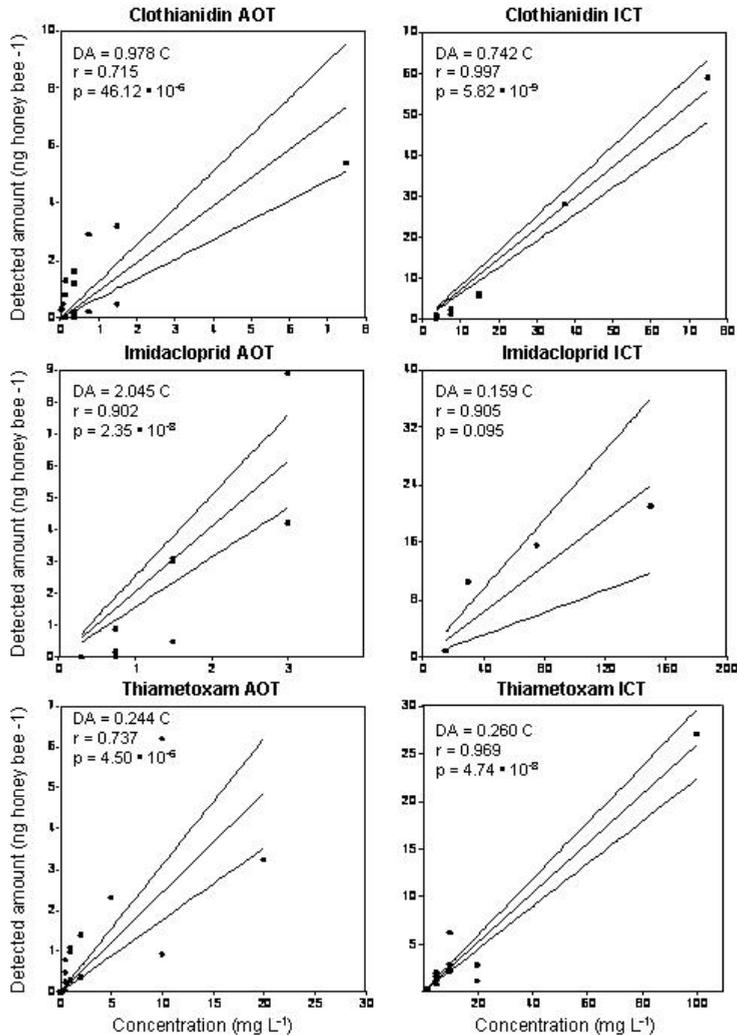

Fig. 1   Linear regression of neonicotinoid amounts recovered from dead honey bees on tested concentrations; 95 % "Working-Hotelling" confidence bands for the fitted lines, Pearson's *r* correlations, and the probability (p) that concentrations (C) and detected amounts (DA) are not correlated are given.

Tests on honey bees taken from colonies belonging to different *A. mellifera* subspecies, although not carried out systematically for every a.i. and every dose/concentration, yielded similar results. The most complete series of data, pertaining to thiametoxam AOT tests on an *A.m.carnica* strain compared with one *A.m.ligustica* colony is shown in figure 2. A Wilcoxon exact test was performed on these data with the statistical package PAST (Hammer, 2001): the probability that the medians of these data are the same is $p = 0.152$.
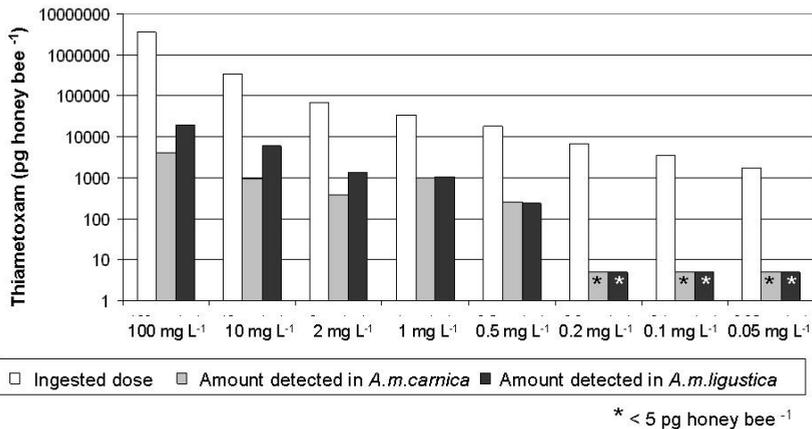
Fig. 2 Amounts of thiametoxam recovered after ingestion of decreasing a.i. doses from dead honey bees that were taken from one *A.m.carnica* and one *A.m.ligustica* hive.

Neonicotinoid amounts recovered from dead honey bees were significantly higher than those recovered from surviving honey bees in each AOT test (figure 3). A Wilcoxon exact test was performed on these data with the statistical package PAST (Hammer, 2001): the probability that the medians are the same is $p = 0.046$. Moreover, half of the alive honey bee samples yielded residue amounts below LOQ.
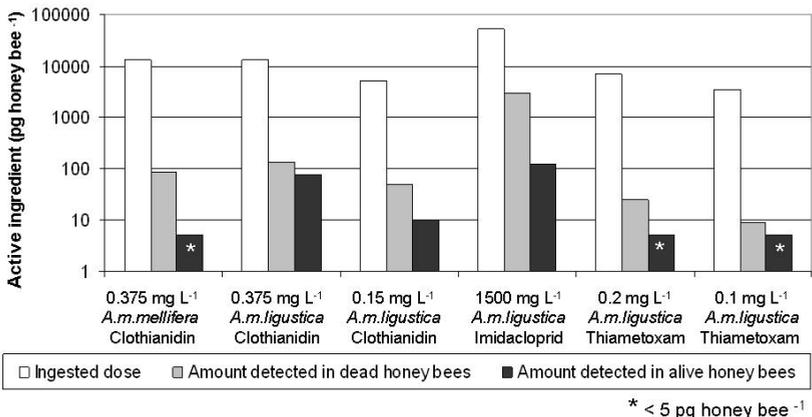

Fig. 3 Amounts of neonicotinoids recovered from dead and living honey bees after ingestion of identical a.i. doses.

The amounts of neonicotinoids recovered from honey bees that died in ICT tests are reported in table 2. Replication number is lower than in AOT tests, but also for ICT most replications were carried out at concentrations that caused intermediate mortalities. The correlation between tested concentrations and a.i. amounts detected in dead honey bees for clothianidin and imidacloprid is better than in AOT tests and the probability that the two variables were not correlated is even lower, while imidacloprid yielded poorer results (figure 1).

Discussion

The poisoning symptoms which were observed during the trials may have reduced a.i. intake by the honey bees mainly in AOT tests and at higher concentrations and may be considered responsible for the somehow irregular results observed. Conceivably that was due to the observed vomiting phenomena and therefore a.i. amounts recovered from dead honey bees which were treated with

field doses were not considered for the calculation of AOT regression lines. Since in ICT honey bees had no opportunity of getting rid of the insecticide through vomiting, results showed a greater regularity and all tested concentrations were used in computations. On the contrary, the ratios between a.i. amounts detected in dead honey bees (DA) and ingested doses (IG) can be calculated only in AOT tests.

The graphs that show regression lines of neonicotinoid amounts recovered from dead honey bees on tested concentrations and the relative 95 % "Working-Hotelling" confidence bands (figure 1) could be used to assess the actual exposure – or a 95 % confidence exposure interval – for honey bees that died on the occasion of a poisoning incident, provided that these honey bees were correctly sampled, stored, handled and analyzed. Such an approach is somehow different from the SRL calculation, which consists in determining the residues present in honey bees after dosing them with one LD_{50} of the insecticide under investigation,[17] and could be readily implemented if dead honey bees were collected and analyzed during the routine determination of AOT LD_{50} of new molecules.

When comparing results of toxicity tests on honey bees performed by different laboratories, substantial differences often emerge and a different genetic response to toxicity tests is sometime advocated to explain such uneven results.[35,36] In the present test, the relevant variation between tested colonies, which belongs to different subspecies and strains, do not appear, but more thorough investigations on response variability between and within subspecies would be welcome.

The rather low DA/IG ratios obtained in the tests could be due to the low stability of the molecules and/or to metabolite formation; both phenomena are well documented[36-38] and probably occurred during the trials. Nevertheless the sharp difference in a.i. amounts recovered from dead and alive honey bees in the same test, even giving allowance for a somehow uneven a.i. intake by different honey bees at the very low concentrations tested, suggests that surviving honey bees were able to better metabolize the insecticide. To better understand the fate of tested neonicotinoids, it would be advisable to determine also the relative metabolites in the dead honey bees, but, to do so, a substantial increase in sample size should be required.

Conclusions

In cases of poisoning incidents, the mere quantitation in dead honey bees of the a.i. involved do not allow to define the real amount of a.i. honey bees were exposed to; therefore it is often impossible to prove that the a.i. is responsible for the observed losses. SRL determination or the implementation of some more refined indexes should be advisable during the normal procedures of authorization for the use of pesticides, so that field survey results could be interpreted correctly.

Acknowledgements

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