

Metabolism of imidacloprid in *Apis mellifera*

S everine Suchail,^{1*} Laurent Debrauwer² and Luc P Belzunces¹

¹INRA, UMR 406 INRA-UAPV Ecologie des Invert br s, Site Agroparc, 84914 Avignon Cedex 9, France

²UMR INRA-ENVT-ENSAT INPT X nobiologiques, 180 Chemin de Tournefeuille, Saint-Martin-du-Touch BP 3, 31931 Toulouse Cedex 9, France

Abstract: Biotransformation of imidacloprid and the appearance of olefin and 5-hydroxyimidacloprid metabolites in the honeybee were studied by HPLC-MS/MS analysis. Honeybees were treated orally with imidacloprid at 20 and 50 $\mu\text{g kg}^{-1}$ bee. Imidacloprid was metabolised relatively quickly and thoroughly. Twenty minutes after the beginning of imidacloprid ingestion, the sum of the residues from the three compounds amounted to only 70% of the actual given dose. Imidacloprid, 5-hydroxyimidacloprid and olefin represented, respectively, 50%, 9% and 8% of the actual ingested dose. Six and 24 h, respectively, after ingestion of imidacloprid at 20 and 50 $\mu\text{g kg}^{-1}$ bee, imidacloprid could no longer be detected in the honeybee. Imidacloprid had a half-life ranging between 4.5 and 5 h and was rapidly metabolised into 5-hydroxyimidacloprid and olefin. Except 5-hydroxyimidacloprid in the 20 $\mu\text{g kg}^{-1}$ treatment, these two metabolites presented a peak value 4 h after ingestion of the 20 and 50 $\mu\text{g kg}^{-1}$ doses. This time fully coincided with the appearance of mortality induced by imidacloprid after acute oral intoxication. These results suggested that the immediate neurotoxicity symptoms are due to the action of imidacloprid, whereas 5-hydroxyimidacloprid and/or olefin are involved in honeybee mortality. In addition, it was likely that the 30% of residues undetected 20 min after intoxication were imidacloprid metabolites, although not 5-hydroxyimidacloprid or olefin. Thus, 5-hydroxyimidacloprid and olefin could not be the major metabolites in the worker bees.

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Keywords: imidacloprid; metabolite; honeybee; metabolisation; LC-MS/MS

1 INTRODUCTION

Imidacloprid is a systemic insecticide that belongs to the neonicotinoid family.¹ This active substance acts as a competitive agonist on the nicotinic acetylcholine receptor (nAChR) of insects.^{2,3} Imidacloprid is extremely effective against a wide variety of insects whereas it appears to be relatively non-toxic to vertebrates.⁴ This selective toxicity of all neonicotinoids is attributable, in general, to the specificity of insect and mammalian nicotinic receptors towards imidacloprid.⁵ In honeybee, imidacloprid exhibits a high toxicity. At 48 h, oral and contact LD₅₀ values are 5 and 25 ng per bee, respectively.⁶ Studies on the acute toxicity of imidacloprid have revealed important characteristics.⁷ (1) After oral exposure, the dose-mortality relation presents a multi-phase profile with ascending and descending phases. (2) The kinetics of mortality are delayed as doses increase. These features suggest that metabolic pathways might be involved in imidacloprid toxicity. (3) Neurotoxicity symptoms appear rapidly, whereas mortality occurs 4 h after acute imidacloprid intoxication and is prolonged during more than 96 h. Thus, imidacloprid

has either a prolonged action or a delayed action, but the symptoms are always immediate. Chronic oral intoxication experiments have shown that imidacloprid induces mortality at concentrations of 0.1, 1 and 10 $\mu\text{g litre}^{-1}$.⁷ These results seem to contrast with those obtained by Schmuck *et al*.^{8,9} who reported that exposure of bee colonies to sunflower honey containing imidacloprid at concentrations of 2, 5, 10 and 20 $\mu\text{g kg}^{-1}$ does not induce any mortality or adverse effects on bee development. This discrepancy in results obtained in chronic toxicity studies may be due to the mode of administration used in the two studies and to the large variability in effects induced by imidacloprid, which depends on the bees and, for a given colony, on its physiological state.⁶

Of the six metabolites tested, only two, 5-hydroxyimidacloprid and an olefin (Fig 1), which are similar in chemical structure to imidacloprid, exhibit an acute toxicity close to that of the parent compound, with a higher toxicity for the latter and a lower toxicity for the former.⁷ Nauen *et al*.¹⁰ reported similar results concerning the toxicity of 5-hydroxyimidacloprid and olefin to the honeybee.

* Correspondence to: S everine Suchail, Laboratoire de Pollinisation Entomophile, UMR 406 INRA-UAPV, Ecologie des Invert br s, Site Agroparc, 84914 Avignon Cedex 09, France

E-mail: suchail@avignon.inra.fr

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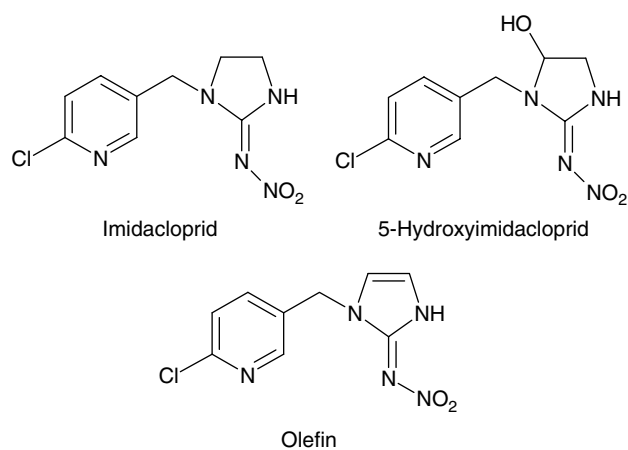


Figure 1. Chemical structures of imidacloprid, 5-hydroxyimidacloprid and olefin.

They showed further that dihydroxyimidacloprid also exhibits toxicity to bees. In other invertebrates, olefin is also highly toxic, being about ten times more active than imidacloprid against the cotton whitefly.¹¹ In pest aphid species, olefin is more toxic than imidacloprid only when it is administered by an oral route, when it is 16 times more active than imidacloprid.¹² Nauen *et al.*¹⁰ reported that the acute toxicity of 5-hydroxyimidacloprid and olefin come from the existence of their nitroguanidine pharmacophore. Metabolites that do not carry this pharmacophore are not toxic to bees. In contrast, in 10-day chronic toxicity studies, all imidacloprid metabolites revealed equal toxicity to bees, but the total dose ingested by the bees was about 3000–100 000 times lower than the doses needed to produce the same effect after acute intoxication.⁷ Toxicity such as that caused by imidacloprid has been already observed previously with other insecticides in honeybees. Fiedler¹³ has studied the chronic toxicity of organophosphorus insecticides to honeybees. For acephate, methamidophos and dimethoate, the cumulated dose resulting in a mortality of 50% was lower than the acute oral LD₅₀ at 24 h.

The discrepancy between the kinetics of mortality and the rapid appearance of neurotoxicity symptoms, together with the insecticidal activity of some metabolites after acute intoxication suggest a combined action of low amounts of toxic metabolites and imidacloprid. Nauen *et al.*¹¹ also suggest a concerted action between toxic metabolites and residues of imidacloprid to explain the long-term residual effectiveness of imidacloprid in plants. Thus, early neurotoxic symptoms could be elicited by imidacloprid itself whereas mortality could be induced by its toxic metabolites, ie 5-hydroxyimidacloprid and olefin, either alone or associated with imidacloprid at low residual amounts. This is in accordance with the action of imidacloprid on honeybee habituation, which is not only age- and dose-dependent but also time-dependent.¹⁴

To understand better the action of imidacloprid in the honeybee, we have studied the biotransformation of imidacloprid in bees after exposure to two

doses close to the LD₅₀. We focused on the two toxic metabolites, 5-hydroxyimidacloprid and the olefin, to discern a possible relationship between the occurrence of these metabolites and the appearance of honeybee mortality. An efficient analytical method was designed especially to determine the residues of these compounds in bees.

2 MATERIALS AND METHODS

2.1 Materials

Imidacloprid [1-(6-chloro-3-pyridylmethyl)-*N*-nitroimidazolidin-2-ylideneamine] and its metabolites, 5-hydroxyimidacloprid [1-(6-chloro-3-pyridylmethyl)-5-hydroxy-*N*-nitroimidazolidin-2-ylideneamine] and olefin [1-(6-chloro-3-pyridylmethyl)-*N*-nitro-4-imidazolin-2-ylideneamine] of analytical quality (98% pure) were obtained from Bayer AG (Leverkusen, Germany).

For all bioassays, worker bees (*Apis mellifera* L.) were captured from honey and pollen combs in a healthy queen-right colony; all drones were discarded. To avoid inter-colony and seasonal variations, all bees were taken from the same colony. Immediately before treatment, bees were anaesthetised with carbon dioxide and kept in cages (10.5 × 7.5 × 11.5 cm) in a dark temperature-controlled chamber at 25 (±1.5) °C and 65 (±5)% relative humidity. Bees were fed a 50% sucrose solution *ad libitum*.¹⁵ In each experiment, three cages of 20 bees were used for each dose of each treatment. Experiments were replicated at least three times.

2.2 Oral application

The honeybees were deprived of food for 2 h before administration of imidacloprid. A stock solution of imidacloprid was prepared in dimethyl sulfoxide (DMSO). This was diluted to the required concentration with DMSO, water and finally 500 g litre⁻¹ sucrose feeding solution so that the final DMSO concentration in the sucrose solutions for the assay and control tests was 1 ml litre⁻¹. The dosing solutions were freshly prepared for each test. Two hundred microlitres of 500 g litre⁻¹ sucrose solution (vehicle) containing imidacloprid at concentrations of 0.5 or 0.2 ng µl⁻¹ were given to groups of 20 bees, which corresponded to a mean individual volume of 10 µl and to doses of 50 or 20 µg kg⁻¹ bee (mean bee weight: 100 mg). Solutions containing 500 g litre⁻¹ sucrose alone were used as controls. In these experiments, bees completely consumed the test solutions. The 50 µg kg⁻¹ dose represented the oral LD₅₀ of imidacloprid in honeybee. The lower dose was also used to examine imidacloprid biotransformation. After consuming the solution containing imidacloprid or the control solution, bees were fed 500 g litre⁻¹ sucrose solution *ad libitum*. The metabolic study was performed only on live worker bees. Honeybees were killed by quick freezing at -80 °C in order to stop their metabolic activity abruptly at specific times: 0, 4, 6, 24, 30 and 48 h after oral intoxication. Time

0 corresponded to the end of total ingestion of the imidacloprid dose by honeybees, which took about 20 min.

2.3 Residue extraction from whole honeybees

For each dose of each treatment, residues were extracted by grinding bees (2 g, corresponding to 20 bees) in methanol + water (1 + 3 by volume; 30 ml). Bee extract was supplemented with Celite (2 g), filtered on a Büchner funnel and concentrated to an aqueous solution by removal of the methanol. For the determination of residues, the aqueous solution was partitioned against dichloromethane (140 ml) using a ChemElut® CE1020 column. The aqueous fraction was concentrated and redissolved in toluene + ethyl acetate (2 ml; 85 + 15 by volume). A second clean-up was performed on a 0.5 g silica gel column by elution with acetonitrile + water (5 ml; 1000 + 1 by volume) at a flow rate of 1 ml min⁻¹. Residues were concentrated and dissolved in acetonitrile + water (2 + 8 by volume). For recovery tests, honeybees (2 g) in methanol + water (1 + 3, by volume) were fortified with a standard solution (0.1 µg ml⁻¹) prepared in DMSO. The recovery rates were determined in fortification experiments in which defined amounts of imidacloprid, 5-hydroxyimidacloprid and olefin metabolites were added to control samples prior to extraction. Solutions containing imidacloprid, 5-hydroxyimidacloprid and olefin were protected from light and stored at 4°C in the dark during the entire procedure.

2.4 HPLC-MS/MS analysis

Fifty microlitres of residues were separated by reversed phase HPLC (C₁₈ column: 150 × 4.5 mm ID; 5 µm particle size) with electrospray MS/MS detection. The flow rates were 1 ml min⁻¹ on the column and 0.15 ml min⁻¹ on the mass spectrometer. The mass spectrometer was tuned by infusing a standard solution of 0.5 mg litre⁻¹ imidacloprid, 5-hydroxyimidacloprid and olefin (dissolved in acetonitrile + water (2 + 8 by volume) containing 0.1 ml litre⁻¹ acetic acid) at a flow rate of 5–10 µl min⁻¹. Details of the gradient for the HPLC-MS/MS measurement samples and the collision energies used were described by Schmuck *et al.*⁸ Residues were identified by their retention times and their *m/z* ratios, where *m* and *z* represented the mass and the charge of the ion, respectively. All compounds were qualified with determination on the ³⁵Cl isotope and confirmation on the ³⁷Cl isotope. Residues were quantified by adding appropriate amounts of derivatives containing ³⁵Cl and ³⁷Cl isotopes. The limit of quantification of 0.5 µg kg⁻¹ was achieved with a minimal signal-to-noise ratio of 3.

2.5 Imidacloprid half-life

Half-life was determined by plotting ln(*I*/*I*₀) against time according to the equation

$$\ln(I/I_0) = -kt,$$

where *I*₀ and *I* represent initial and residual imidacloprid concentrations. Half-life was calculated with the equation

$$t_{1/2} = (\ln 2)/k,$$

where *t*_{1/2} is the half-life and *k* is the apparent elimination constant.

3 RESULTS

After oral ingestion of imidacloprid, early symptoms of poisoning included hyperactivity and tremors. After several hours, these symptoms gradually disappeared and the worker bees became hypoactive. After extraction, residues were separated by HPLC-MS/MS. The retention times of olefin, 5-hydroxyimidacloprid and imidacloprid were 4.5 min, 5.5 min and 9 min, respectively. Samples were fortified with imidacloprid and its metabolites at 10 mg kg⁻¹. Recovery (mean ± SE) was about 98.6 (±4.1)% for imidacloprid, 85.2 (±6.3)% for 5-hydroxyimidacloprid and 78.9 (±11.9)% for olefin.

The biodegradation of imidacloprid and the appearance of 5-hydroxyimidacloprid and olefin in honeybees were followed after oral application of imidacloprid at the doses of 50 or 20 µg kg⁻¹ bee (Figs 2 and 3). The true doses administered to honeybees were 48.2 and 19.6 µg kg⁻¹ bee and were determined by HPLC with UV detector set at 270 nm.

Imidacloprid was metabolised relatively quickly and thoroughly. After total ingestion of 50 µg imidacloprid kg⁻¹ bee, the sum of imidacloprid, 5-hydroxyimidacloprid and olefin residues represented 67% of the actual dose given. Imidacloprid, 5-hydroxyimidacloprid and olefin represented respectively, 50%, 9% and 8% of the actual dose. After 6 h, only 10.5% of imidacloprid were detected in honeybees and after 24 h, it could not be detected. Imidacloprid had an elimination half-life of 4.5 h and was rapidly metabolised into 5-hydroxyimidacloprid and olefin. These two metabolites reached a peak value 4 h after oral intoxication. Olefin and 5-hydroxyimidacloprid represented respectively, 9.5% and 13% of the initial dose 4 h after intoxication. For both metabolites, the amount of residues started to decrease 4 h after exposure and represented approximately 5% of the ingested dose 48 h after exposure.

When the individuals were exposed to a dose of 20 µg of imidacloprid per kg of bee, the metabolism kinetics of imidacloprid were similar. Twenty minutes after total ingestion, imidacloprid represented 55% of the actual dose and after 6 h only 3% of residues were detected. The imidacloprid half-life was 5 h after intoxication with a dose of 20 µg kg⁻¹ bee. Olefin metabolite presented a peak value 4 h after ingestion time at which time it represented 14% of the actual ingested dose. 5-Hydroxyimidacloprid had a different kinetic appearance. It appeared immediately during or after, the ingestion period

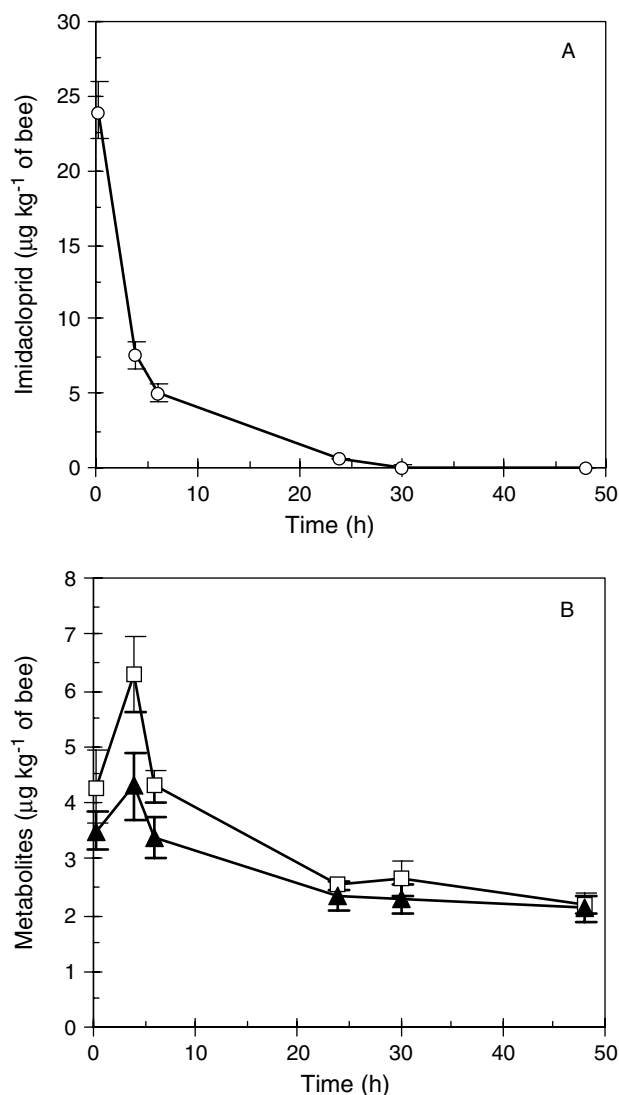


Figure 2. Biotransformation kinetics of imidacloprid after oral exposure to 50 µg kg⁻¹ bee. A: Imidacloprid degradation. B: Appearance of (□) 5-hydroxyimidacloprid and (▲) olefin. Data represent the means of three experiments performed in triplicate ±SD.

and, at the beginning of the biotransformation kinetics, represented about 11% of the initial dose. Both compounds decreased with time and, 4 h after intoxication, amounted to about 14.3% and 8.7% of the initial ingested dose for olefin and 5-hydroxyimidacloprid, respectively.

4 DISCUSSION

Earlier toxicological studies of imidacloprid in the honeybee show a discrepancy between mortality kinetics and the rapid appearance of neurotoxicity symptoms.⁷ Moreover, neurotoxicity symptoms appeared within 10 min of intoxication, whereas 5-hydroxyimidacloprid and olefin, two metabolites having toxicity close to that of imidacloprid⁷ increase in the bee during the initial 20 min of imidacloprid intoxication. However, they are in low amounts compared with imidacloprid and present a peak value 4 h

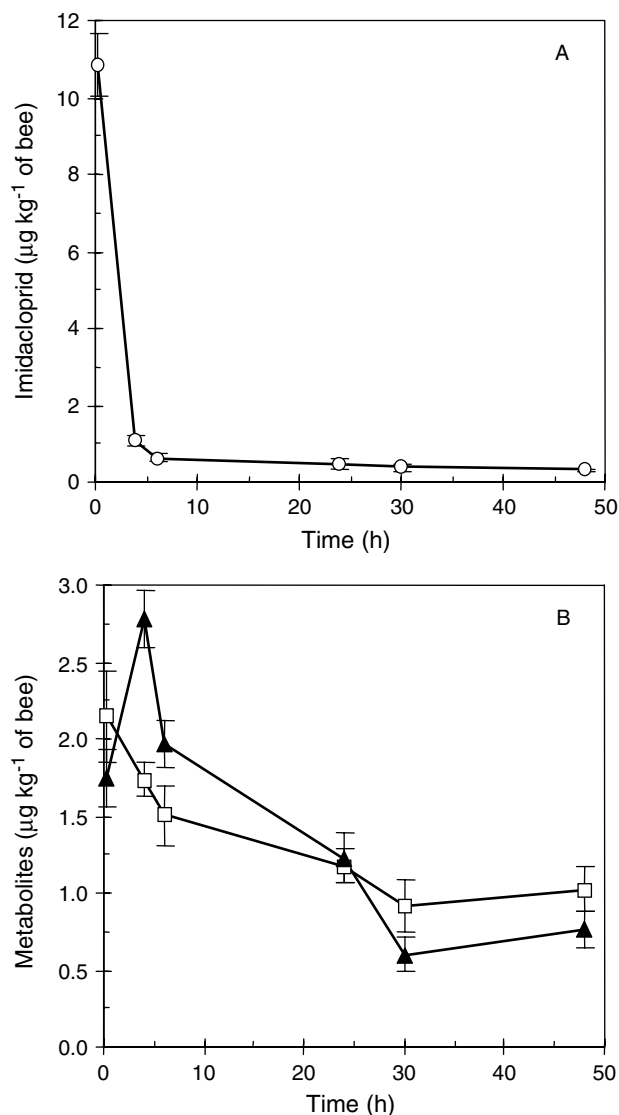


Figure 3. Biotransformation kinetics of imidacloprid after oral exposure to 20 µg kg⁻¹ bee. A: Imidacloprid degradation. B: Appearance of (□) 5-hydroxyimidacloprid and (▲) olefin. Data represent the means of three experiments performed in triplicate ±SD.

after intoxication, when imidacloprid has drastically decreased. This suggests that the early neurotoxicity symptoms are induced preferentially by imidacloprid whereas these two toxic metabolites are involved in the bee mortality along with or without residual imidacloprid.

Residue tests performed 20 min after intoxication with the doses of 20 or 50 µg per kg bee showed that imidacloprid can be detected at only 74.5% and 67% of the initial dose ingested by the honeybees, respectively. In other words, about 30% of the residues remained undetected in honeybees. These results imply that either the residue extraction method is not efficient enough or that bees rapidly excrete imidacloprid in its native or metabolised form. It is less probable, however, that the failure to detect 30% of residues is due to the low efficiency of the extraction method because the yield of this method is about 98%. In addition, this cannot be due to

the residue excretion by bees because no sign of this was observed 20 min following oral intoxication. Normally, under these experimental conditions, honeybees excrete residues in faeces at least 12 h after ingestion of active substance. Thus, it is very likely that these 30% of residues might be imidacloprid metabolites other than 5-hydroxyimidacloprid and olefin. In this case, 5-hydroxyimidacloprid and olefin could not be the major metabolites in the worker bees.

Several methods for the determination of imidacloprid have been published. They involve determination by enzyme-linked immunosorbent assay (ELISA),^{16,17} analysis by liquid chromatography coupled with diode-array detection¹⁸ or mass spectrometry,^{19,20} and a combination of liquid chromatography and supercritical fluid extraction to increase residue recovery.²¹ However, HPLC-MS/MS is an effective, fast tool for obtaining very useful information on the nature and the quantity of the compounds analysed. This analytical method makes it possible to separate and identify imidacloprid, 5-hydroxyimidacloprid and olefin, and to decrease the limit of quantification (LOQ) of these compounds. This HPLC-MS/MS analytical technique combines sensitivity and selectivity, and allows low LOQ for imidacloprid, 5-hydroxyimidacloprid and olefin even with complex matrices. LOQ depends on sample origin. In sugar beet leaves and roots the limit of detection (LOD) of imidacloprid and its metabolites is 10 µg kg⁻¹ fresh weight.²² In nectar and beebread, a LOQ of 10 µg kg⁻¹ and a LOD of 3 µg kg⁻¹ has been reported by Wallner.²³ In honeybees, this method has given a LOQ of 0.5 µg kg⁻¹ for imidacloprid, 5-hydroxyimidacloprid and olefin. Hence, the LOQ obtained for imidacloprid and its two toxic metabolites makes it possible to study metabolism at doses close to the sub-lethal level so as to understand the mode of action.

The present study showed that honeybees are able to rapidly metabolise imidacloprid through a process that reduces exposure to the parent compound and, in most of cases, its toxicity. However, the appearance of 5-hydroxyimidacloprid and olefin, two metabolites toxic at low doses in chronic exposure and at higher doses in acute exposure, coincides with the appearance of mortality induced after acute oral intoxication by imidacloprid. These results strongly suggest that 5-hydroxyimidacloprid and/or olefin contribute to extending the action of imidacloprid in honeybees. Moreover, this study also suggests the existence of other imidacloprid metabolites. It would be interesting to characterise all imidacloprid metabolites in honeybees after oral intoxication by imidacloprid to obtain the whole metabolic profile. Furthermore, locating all the residues in different compartments of honeybees could help us to understand the mode of action of imidacloprid by correlating the pharmacokinetics and the pharmacodynamics of imidacloprid in honeybees.

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