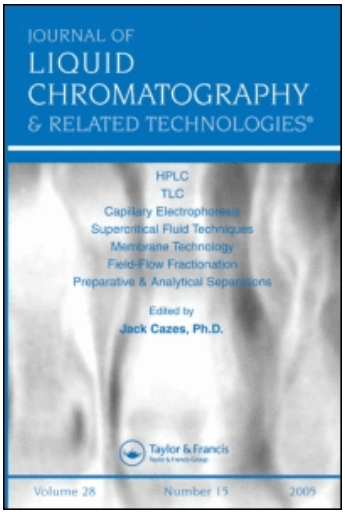

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EVALUATION OF THE TOXICITY OF IMIDACLOPRID IN WILD BIRDS. A NEW HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC) METHOD FOR THE ANALYSIS OF LIVER AND CROP SAMPLES IN SUSPECTED POISONING CASES

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ABSTRACT

Several accidents were reported in France, in domestic and wild animals, involving the potential ingestion of imidacloprid-coated seeds. Imidacloprid is a new insecticide which acts as a nicotinic blocker. Since there was very limited published information regarding this compound, its toxicity to wild birds, and potential routine analytical techniques, this study was designed to set up and validate a method for the detection and quantification of imidacloprid and its primary metabolite, 6-chloronicotinic acid, in tissues and organs of affected animals. A high performance thin layer chromatography (HPTLC) technique was developed to quantify both compounds. The method is repeatable, reproducible, and sensitive enough to investigate potential poisoning cases (limits of quantification between 0.25 and 0.5 µg/g).

The technique was also applied successfully to birds found dead after a known exposure to the compound and indicates that imidacloprid accounts for the majority of the toxic residues detected in the liver of affected pigeons.

INTRODUCTION

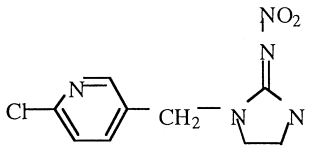
Imidacloprid is a potent new insecticide which is used primarily as a systemic soil insecticide and seed treatment in France and several other countries around the world.[1,2] It acts on the central nervous system, causing irreversible blockage of the post-synaptic nicotinic receptors.[2] Very limited references are available on this compound, mostly on its insecticidal properties[3] and biodegradation in soil.[4] Its toxicity in animals has been investigated and it appears acutely toxic to birds, with oral lethal dose 50 (LD 50) as low as 25-30 mg/kg in canary, pigeons, and Japanese quails.[2,5] The primary purpose of this study was to establish and validate a simple analytical technique for imidacloprid in animal tissues, since very limited information on this product is available to date. The second objective was to analyze animal tissue samples for imidacloprid and its major metabolite 6-chloronicotinic acid,[2] and to set up a routine analytical procedure to investigate accidental exposures in birds.

EXPERIMENTAL

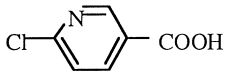
Extraction was based on previously published methods.[4] For routine purposes, it is essential to have efficient and rapid extraction procedures. For that reason, the technique was adapted as follows, with ultra-pure HPLC-grade reagents.

Reagents and Chemicals

One g of crop, gizzard content, or liver tissue was weighed and ground, and then extracted with 5.0 mL of dichloromethane and 1.0 g of sodium sulfate. The sample was centrifuged at 2,000 rpm for 5 min and the supernatant was separated. The solid residue was extracted twice similarly. Both supernatant fractions were combined, filtered, and evaporated at 40°C under a nitrogen flow and re-solubilized with 1.0 mL of methanol (concentration coefficient 1.0). Spiked samples were prepared to determine the percentage of recovery as follows: 1.0 mL of a 2% solution of imidacloprid was added to 1.0 g of biological sample and evaporated under a nitrogen flow before undergoing the usual extraction procedure.



Imidacloprid



6-Chloronicotinic acid

Figure 1. Substances studied.

The residual sample was extracted with acidified dichloromethane (0.1% H_2SO_4 in dichloromethane) and extracted twice with 10 mL of solvent. After evaporation to dryness under nitrogen stream, the residue was dissolved in 1.0 mL of acidified methanol (0.25% H_3PO_4). The samples were analyzed by HPTLC.

Standard solutions were prepared in methanol using the following substances: imidacloprid and 6-chloronicotinic acid (see Fig. 1). Chloronicotinic acid is considered to be the major metabolite of imidacloprid in animals, with a higher residence time in the liver and, therefore, it is recommended as a tracer of the parent compound.[2] Calibration curves were prepared after dilution (1.0 to 10.0 µg/mL). Imidacloprid standard was obtained from CIL (Ste Foy la Grande, France) and 6-chloronicotinic acid was purchased from Sigma Chemicals (St Quentin Fallavier, France). The purity of the chemicals was >99% in both cases.

HPTLC Analysis

Sampling was performed automatically, using an Automatic TLC Sampler III (ATSIII-Camag, Basel, Switzerland). Ten µL of each sample/standard was sprayed as a thin band (6 mm x 0.5 mm) with pressurized nitrogen gas (6,000 HPa) at a distance of 1.0 cm from plate edges. Samples were separated by a 3-5 mm space. Usually, 18-20 samples can be sprayed on one 10x20 cm-plate.

The plates used were nano-HPTLC Si60 F254 (Merck Nogent sur Marne, France) 10 x 20 cm. A single batch number was used throughout the validation procedure.

After spraying, the plates were developed with chloroform-acetone-methanol (23:1:1) for imidacloprid determination alone (elution 1) and a mixture of acidified chloroform-ethylene dichloride-methanol (13/2/10) for imidacloprid and chloro-nicotinic acid determination (elution 2). A small elution chamber was saturated for 5-10 min with the solvent mixture and elution required less than 20 min (8 cm development). After development, the plate was removed and allowed to dry for 5-10 min.

Densitometry was performed with a TLC3 scanner (Camag) driven by the Cats (Camag) software. A pilot track (standard) was read first at 275 nm (deuterium lamp). This wavelength was selected in preliminary experiments because it corresponded to an absorption maximum for both imidacloprid and its metabolite. Peaks were then integrated. A UV spectrum (220-380 nm) was obtained for each detected peak on the plate. Each substance was then characterized by its R_f value and UV spectrum. For each substance, three replicates per plate were analyzed and five plates were examined to check the reproducibility of the R_f values measured. Standard UV spectra were recorded in a spectrum library for future identification purposes. Spectra of standards and samples (unknown or spiked) were then compared by means of the Cats Camag[1] software.

Calibration curves were prepared, using five points (1.0-10.0 µg/mL). For each point, three measurements were made to improve repeatability of the procedure. Reproducibility was checked with three plates on three different days. For each day, six points per value were used, data were averaged, and the calibration curve was determined by means of a linear regression model (least square regression). Extraction (percent recovery) was determined on three series of five points (as for the calibration curve). Dilutions of a 100 µg/mL standard solution in methanol were used to prepare spiked samples.

Biological Samples

Several samples of wild birds (mainly pigeons and partridges) were submitted to the laboratory after a known exposure to coated-seeds (animals found dead in a barn, near an open bag of coated seeds, or found dead in treated fields). These birds are designated hereafter as “exposed.” Necropsy was performed and revealed the macroscopic evidence of ingestion of treated seeds, since pink-red colored cereal seeds were noted in the crop of all exposed birds. When these seeds were detected in the crop content, the analytical procedure included the analysis of the crop and the gizzard content, as well as the liver,

when available, to check for the presence of either imidacloprid or its metabolite. Specificity was checked on gizzard or crop or liver of control birds (pigeons and partridges) not suspected of imidacloprid exposure (mainly birds killed during hunting).

Statistical Analysis

Linearity was determined with the least square regression method. Slopes were compared by means of ANOVA of the regression and Y-intercepts were compared by means of a Student's t-test (0.05 and 26 degrees of freedom).

RESULTS

Imidacloprid and 6-chloronicotinic acid (Fig. 1) can be separated on the same plate. The mean R_f was 0.87 ± 0.02 (coefficient of variation, CV = 2.3%, n = 5 replicates) for imidacloprid and 0.68 ± 0.03 (CV = 4.4 %) for chloronicotinic acid (elution 2).

When both standards were mixed together, the results were identical (same separation). When imidacloprid was analyzed separately (elution 1), the mean R_f was 0.34 ± 0.07 (CV = 2.0%).

Specificity

Specificity of the method was determined with blank liver and untreated cereals. Fig. 2 presents chromatograms of a) blank liver extract and b) 2 mg/kg imidacloprid and chloronicotinic acid spiked samples. Fig. 3 presents the solid-phase UV spectrum recorded for each standard and two impurities found in liver extracts (R_f 0.55 and 0.82).

Extraction

Extraction was performed on cereal and liver samples. Imidacloprid was analyzed in all samples and 6-chloronicotinic acid was analyzed only in the liver. Table 1 summarizes the values obtained with these samples.

Percent extraction varied between 68.8% and 75.4% for imidacloprid and between 65.1% and 94.3% for its metabolite, for spiked samples from 1.0 to 10.0 mg/kg (see Table 1).

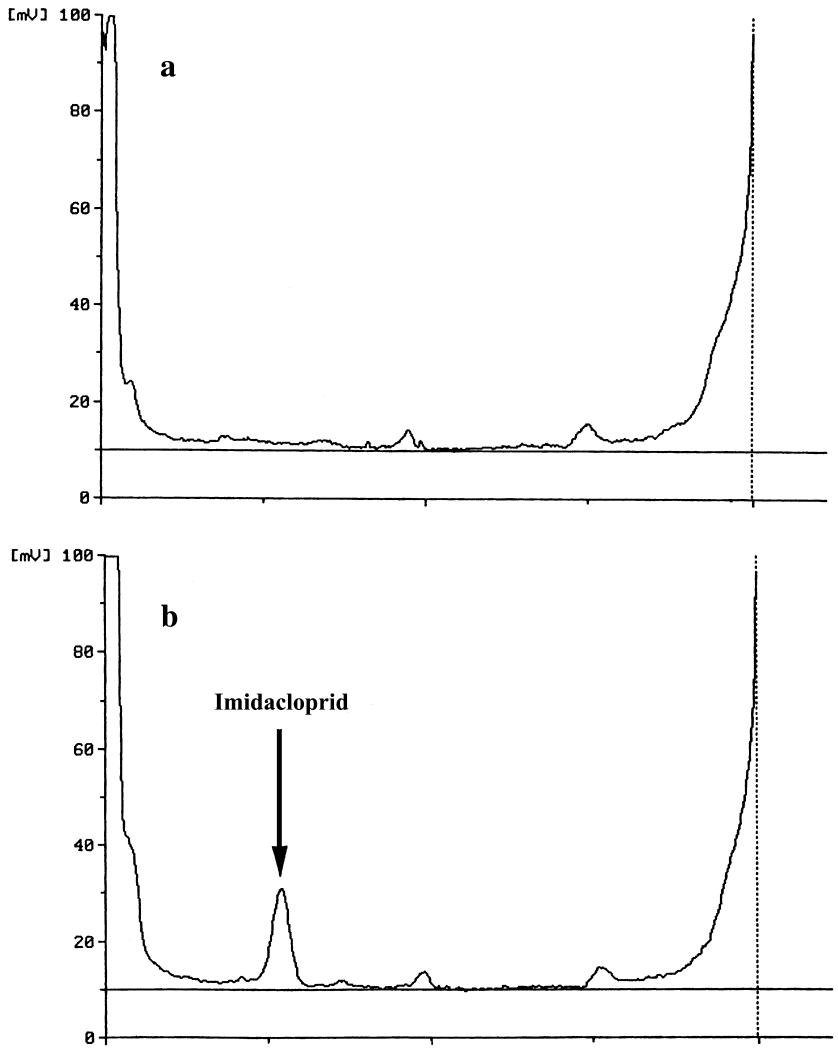


Figure 2. Chromatograms of a) blank liver extract and b) 2 mg/kg imidacloprid and chloronicotinic acid spiked samples.

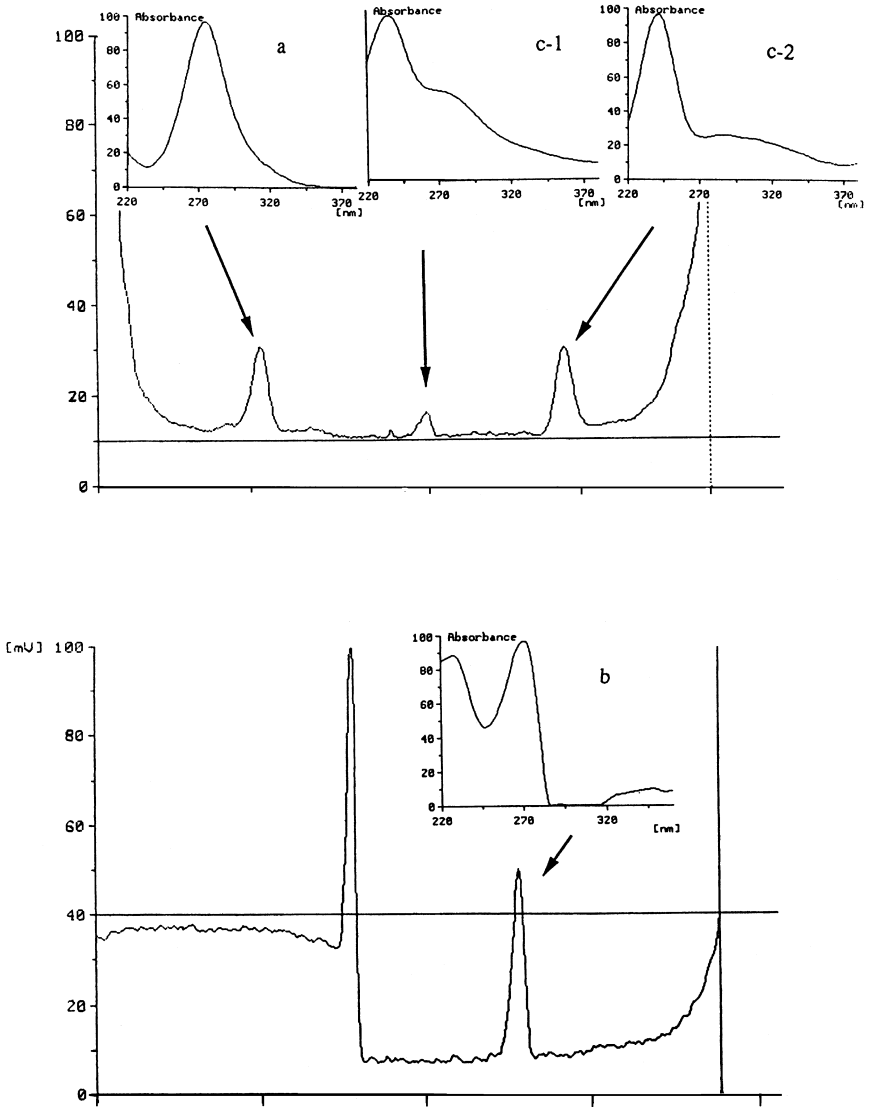


Figure 3. Solid-phase UV spectrum recorded for each standard and for two impurities found in liver extracts (R_f 0.55 and 0.82).

Table 1

Percent Recovery of Imidacloprid (Imida) and 6-Chloronicotinic Acid (6CA) in Liver Samples

Analyte	1.0 mg/kg	2.0 mg/kg	4.0 mg/kg	8.0 mg/kg	10.0 mg/kg
Imida	75.4	77.1	70.9	68.8	68.9
6CA	94.3	75.4	71.1	75.2	65.1

Table 2

Validation of the Analytical Procedure for Imidacloprid and 6-Chloronicotinic Acid

Parameter	Imidacloprid	6-Chloronicotinic Acid
Detection Limit (µg/g)	0.3 (gizzard)/0.5 (liver)	0.5 (liver)
Quantification Limit (µg/g)	0.5 (gizzard)/1.0 (liver)	1.0 (liver)
Extraction	68.8%-77.1%	65.1%-94.3%
Repeatability (2µg/g)	3.3% (n = 6)	9.3% (n = 6)
Coefficient of Variation: Linear Equation (spiked samples)	97.7 C + 29.7	92.6 C + 101.7
Linearity (r^2)	0.999	0.999

Detection Limit

The detection limit was determined according to Caporal-Gautier, et al.,[6] in compliance with the European Union validation procedures (Note 844/87).

Blank samples of cereals or chicken liver were analyzed five times and the limit of detection (determined as the mean noise level + 3 SD) was 0.1 mg/kg in gastrointestinal tract and 0.3 mg/kg in liver samples for imidacloprid. For chloro-nicotinic acid, the LOD was 0.5 mg/kg in liver samples.

Quantification

Linearity, repeatability, reproducibility

The limit of quantification was determined[6] as mean noise level + 10 SD and found to be 0.5 mg/kg in crop/gizzard content and 1.0 mg/kg in liver samples for imidacloprid. It was determined as 1.0 mg/kg for 6-chloronicotinic acid in liver samples.

Repeatability of the method was tested for 2.0 ppm standards of imidacloprid or its metabolite (see Table 2). Repeatability was excellent for imidacloprid (CV < 5%) and good for 6-chloronicotinic acid (CV < 10%) and the reproducibility of the method was excellent: CV < 5 % over three days for both compounds.

Linearity was determined with five points (1.0, 2.0, 4.0, 8.0, and 10.0 ppm) and r^2 were always > 0.99. The Y-axis intercepts were statistically not different from 0. Calibration curves for standards and spiked samples (from 1 to 10 mg/L theoretical value) are presented in Fig 2. Each point was determined as the mean of three replicates. CVs were always below 5% except for one point (1.0 mg/kg spiked sample: CV = 8.3%). A polynomial regression model (2nd order) was tested and found to be more accurate (r^2 = 1.0) for both compounds. With this model, the Y axis intercept was not statistically different from zero. The Y-intercepts were found to differ significantly between standards and spiked samples, while the slopes did not significantly differ.

Application to exposed animals

This technique was applied to wild birds known to be exposed to the product, since they were found dead with noticeable amounts of coated seeds in the crop. When available, liver samples were tested for imidacloprid or its metabolite. Table 3 summarizes values detected in crop/gizzards of pigeons and partridges as well as liver concentrations of imidacloprid in control animals killed (hunting) and in animals exposed and found dead. There was a significant difference between liver concentration of imidacloprid in partridges and pigeons (Mann-Whitney U test, $p<0.05$). Chloronicotinic acid was detected below the limit of quantification in the liver samples examined.

DISCUSSION

Imidacloprid is a new insecticide. It is used as seed treatment for cereals, sugar beets, corn, rice, and sunflower.[1] So far, there is no report of poisoning in animals, but it was expected to occur in seed-eating birds such as partridges, considering the amount of coated seeds they can eat and the acute toxicity of imidacloprid in many bird species (quails and pigeons for instance).[5]

Table 3

Median Concentration and Range of Imidacloprid in Crop, Gizzard, and Liver Samples of Control Animals and Birds Found Dead and Suspected of Poisoning[a]

Group of Birds	Crop Conc.	Gizzard Conc.	Liver Conc.
Exposed Partridges (n = 12)	**11.4** (8.3-70.0)	**4.1** (2.2-10.5)	**1.2** (1.0-1.6)
Control Partridges (n = 20)	**<0.2** (<0.2-3.5)	**<0.2** (<0.2-3.1)	**<0.2**
Exposed Pigeons (n = 7)	**16.3** (7.6-79.0)	N.A.[b]	**1.7** (0.6-3.1)
Control Pigeons (n = 6)	**<0.2** (<0.2-0.6)	N.A.[b]	**<0.2**

[a] Values in μg/g, wet weight. [b] N.A.: Not available.

Our attention was drawn to this compound after a series of reports of unusual death of partridges. The birds all appeared weak and were reluctant to move and unable to fly. Dead animals all contained coated seeds in their crop. These signs were compatible with imidacloprid poisoning, since it is a nicotin-like insecticide.[2] Based on our experience, it was decided to set up and validate a method to analyze for imidacloprid, since no routine technique was available. Our laboratory has been using HPTLC for several years, and this technique is simple, rapid, and not expensive.[7] We developed two different development procedures because our preliminary results indicated that imidacloprid was the primary compound detected in acute exposure and also because the method used for the simultaneous determination of chloronicotinic acid and imidacloprid usually resulted in more interferences, especially with regards to poorly refrigerated liver samples (data not shown). Although these interferences did not alter the results for any of the compounds tested, it appeared interesting to validate both development procedures for routine purposes. Our results with imidacloprid indicate that this method is repeatable, sensitive, specific, and reproducible, based on the criteria selected for drug analysis.[6] HPTLC may sometimes suffer from poor reproducibility, mainly because of external conditions (room temperature, humidity, plates, saturation of the elution chamber).[7,8] However, despite these obvious potential problems, we did not experience this problem with this method.

It is also often considered that quantification is better described by polynomial regression than linear regression, based on physical phenomena occurring within the plate.[8] In our experience, polynomial regression was the

best fit to the calibration curves obtained, but it seems preferable, for practical purposes, to use linear regression, since the results were excellent ($r^2 > 0.99$ in all cases). Extraction could be improved but, for daily practice, we use spiked samples to create a "spiked calibration curve" from 1.0 to 10.0 ppm to take into account this limited extraction. These spiked samples are analyzed with samples submitted to our laboratory, on the same plate.

It also appears desirable, since calibration curves on spiked samples and standards have different Y-intercepts, to use calibration curves obtained with spiked samples, to account for extraction and Y-intercept biases.[6] Finally, analysis by use of a UV spectra is a good way to identify the substance and offers greater potentialities than a standard one-wavelength HPLC analysis.[7] A high performance liquid chromatographic method has been recently published for both imidacloprid and 6-chloronicotinic acid[9] in samples of protective garments and absorbent pads applied on workers exposed to imidacloprid during spraying. Urine was analyzed for chloronicotinic acid. The authors reported better extraction coefficients (89-110%) in these matrices, with a very similar extraction procedure (methylene dichloride was the extracting solvent). We consider that the liver is a more complex matrix than clothes and pads with many potential interfering compounds and that the distribution of imidacloprid within the organ is responsible for our lower recovery. Interestingly, Calumpang and Medina[9] did not report any difficulty in chloronicotinic acid extraction from the urine, while we had to acidify the liver extracts in order to get any detectable amount of the metabolite.

The biological samples analyzed here were quite interesting. First of all, the differences observed between exposed and control individuals were quite high and significant, and there was no prior report of poisoning with this new insecticide, especially with confirmatory analysis.

Second, the liver appears as the organ of choice to confirm a suspected poisoning case. Experimental poisoning also indicated that the liver and the kidney contained the highest and most reliable concentrations of imidacloprid.[5] Indeed, crop and gizzard concentrations were quite inconsistent and could lead to false conclusions. It could happen, for instance, that the birds would regurgitate part of the product as a consequence of the toxicity and the remaining crop concentration would be low, even though the animal was actually poisoned.[10] Conversely, some birds may be affected for several days before dying, and residual concentrations in the gastro-intestinal tract would be too low to confirm poisoning.

Third, our results are field data and indicate that imidacloprid may cause some deaths, despite its repellent effect.[5] The repellent effect of imidacloprid is supposed to occur after the birds have tasted the product, suffered from it to a certain extent, and also because the birds would recognize the bright red-colored seeds afterwards and be able to associate the color with the effects.[5] This may

be true, but some birds in field situations may still overcome the repellent effect when food is poorly available.

Finally, it seems that partridges may be even more susceptible than pigeons, since the exposed birds had between 1.0 and 1.5 mg/kg imidacloprid in the liver, while pigeons sometimes had 3.0 mg/kg imidacloprid in the liver and there was a statistical difference between both species as far as liver concentrations were concerned (Mann Whitney test, p=0.05). The liver concentrations were surprisingly low, considering data obtained under experimental conditions (Dr Pflüger, Bager AG, personal communication), since it is considered that poisoning is usually associated with liver concentrations above 5 mg/kg. Our values never reached that concentration. It should be remembered that experimental birds were fed only coated seeds, which may result in higher concentrations, and that only quails were used. Therefore, species differences may account for these discrepancies. However, our results, together with the data from experimental poisoning, indicate that our LOD and LOQ values for imidacloprid are low enough to investigate suspected poisoning cases.

Unfortunately, our results with chloronicotinic acid are not as straightforward. It is likely that the extraction procedure should be modifed to improve the recovery and the detection limit. However, it should be pointed out that in the birds found dead in the fields, death occurred apparently rapidly (seeds were found mainly in the crop and rarely in the gizzard), and imidacloprid was detected mostly as the parent compound in the digestive tract and in the liver. The 6-chloronicotinic acid moiety was only detected at trace levels (between the detection limit and the quantification limit, i.e., between 0.5 and 1.0 µg/g). Consequently, for diagnostic purposes, it seems that analysis should be directed primarily towards imidacloprid and use the elution solution 1 only.

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