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Distribution of [^{14}C]Imidacloprid in Sunflowers (*Helianthus annuus* L.) following Seed Treatment

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Imidacloprid, a neonicotinic insecticide, has been used as a seed dressing (Gaucho) to protect crops against soil and aerial insects. However, French beekeepers observed abnormal behavior of bees foraging on sunflowers and suspected a link between the imidacloprid seed treatment and the observed bee syndrome. This work studies the distribution of [^{14}C-*imidazolidin*]imidacloprid (1 mg/seed) in three stages of Gaucho-treated sunflowers grown in an outdoor lysimeter. Plants absorbed <10% of [^{14}C]-imidacloprid spiked on seeds, and 75% of that absorbed radioactivity was found in cotyledons. Concentrations in the upper leaves were 20 times lower than in the first leaves. From the extracted radioactivity, imidacloprid accounted for 50% and metabolites for the other 50%. Four major metabolites can be detected, in variable concentrations, among which the hydroxy- and olefin-imidacloprid have toxicities equivalent to that of imidacloprid. In pollen, concentrations of imidacloprid were 13 ng·g^{-1}. Thus, imidacloprid residues from Gaucho seed treatment contaminated sunflower pollen, involving the translocation of imidacloprid within the plant.

KEYWORDS: ***Helianthus annuus*; sunflowers; imidacloprid; insecticide residues; bees**

INTRODUCTION

Since its introduction 10 years ago as an insecticide for crop protection (*1*), imidacloprid (**Figure 1a**) has become the most widely used product. This is primarily due to the novel mode of action of this chloronicotinyl compound, which is an agonist of the nicotinic acetylcholine receptor (nAChR) (*2*, *3*). Before the commercialization of imidacloprid, nAChR was a very minor target for insecticides. Thus, there is no cross-resistance between imidacloprid and the major insecticide groups (carbamates, organophosphates, and pyrethroids) (*4*).

Imidacloprid acts on numerous insects by contact and ingestion (*1*). Treatments are applied by spraying, soil granules, or seed dressing. Imidacloprid is the main insecticide used as a seed dressing (Gaucho). This treatment protects seeds and roots against soil insects (*5*, *6*) and, due to the systemicity of imidacloprid in plants, it also efficiently protects aerial parts against insect pests such as aphids (*7*). Seed dressing allows a considerable decrease in the dosage rate compared with conventional spraying. Currently, the seeds of many different crops are treated with Gaucho, for example, maize, cereals, sugar beet, and sunflowers.

Since 1994, French beekeepers have observed losses in honeybee foragers from April to July and a dramatic decrease in the sunflower honey crop (*8*). It has been hypothesized that this phenomenon is linked with abnormal behavior of honeybees foraging on sunflowers. After the sunflower blossoming period, hive activity returns to normal. However, in the spring, which follows exposure to sunflowers, the development of the brood is disturbed, resulting in a decrease in the hive activity and the number of bees.

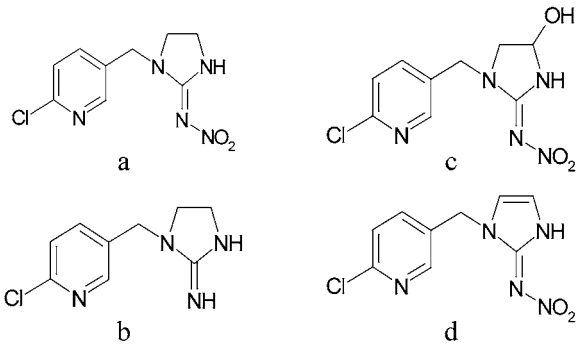

Figure 1. Structures of imidacloprid and some metabolites: (**a**) imidacloprid; (**b**) guanidine analogue; (**c**) 4-hydroxy-imidacloprid; (**d**) 4,5-deshydro-imidacloprid (olefin).

French beekeepers suspected the use of imidacloprid dressing on sunflower seeds to be the cause of the "French honeybee sunflower syndrome" (*9*). Their suspicion was strengthened by the increase in this behavioral disruption associated with an increase in the use of imidacloprid in sunflowers. In this context, several points remain to be investigated, two of which are crucial: (i) the amount of imidacloprid that needs to be ingested by honeybees to produce the observed sublethal effects and (ii) the degree of probability for this behavior to occur when sunflower seeds are treated with imidacloprid.

In this study, we focused on the risk of exposure and on the fate of imidacloprid during the vegetative and blossoming

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periods in sunflower plants grown outdoors from ([^{14}C]-imidacloprid)-Gaucho-dressed seeds. The fate of imidacloprid in sunflowers has already been explored by Schmuck et al. (*10*) with radiolabeled imidacloprid in controlled growing conditions and by Bonmatin et al. (*11*) and Lagarde (*12*) in fields with commercial treatment. Our approach combined radioactive labeling (to exhaustively follow the fate of imidacloprid residues) with both controlled environment and outdoors conditions to approach the field conditions. Imidacloprid metabolism was analyzed during the growth of the sunflowers, as its metabolism has already been described in some plants (*13*) but not in sunflowers. Among known pathways, the metabolism in the imidazolidin ring is particularly important in risk assessment because its products, 4-hydroxyimidacloprid and its olefin analogue (**Figure 1c**,**d**), are suspected to be toxic to bees (*14*).

MATERIALS AND METHODS

Chemicals. Unless otherwise stated, chemicals were purchased from Sigma (Saint-Quentin Fallavier, France). [U-^{14}C-*imidazolidine*]Imidacloprid (specific activity = 26.8 mCi·mmol^{-1}, radiochemical purity >97% as determined by radio-HPLC) was obtained from Institute of Isotopes Co. (Budapest, Hungary). Non-radiolabeled imidacloprid, Pestanal quality, was purchased from Cluzeau (Bordeaux, France). Bayer kindly provided the imidacloprid metabolites.

Plant Material and Sampling. *Treatments.* Sunflower seeds, cv. Albena RM, coated with Gaucho 70 WS and 0.25 L/Q of Quinolate Pro FL (1 mg of imidacloprid active ingredient/seed), were provided by Bayer. Amounts of 5 or 7.5 μCi of [^{14}C]imidacloprid dissolved in 40 μL of acetone were deposited on the dressing (20 μL to each seed face). Seeds were then dried for 5 min under ventilation.

Culture and Sampling. (a) Cabinet Experiments. Gaucho-treated sunflower seeds (5 μCi of [^{14}C]imidacloprid/seed) were sown in 1 L pots. They were left to grow for 1 month in a climate-controlled cabinet at 25/20 °C (day/night) with a 16 h photoperiod. Pairs of cotyledons and leaves were then collected. Stems were cut into sections above leaf insertions. Undeveloped tops of plants were collected as apexes.

(b) Lysimeter Experiments. Gaucho-treated sunflower seeds (7.5 μCi of [^{14}C]imidacloprid/seed) were individually sown at a depth of 2 cm in plastic pots containing a compost−sand−soil mixture (1:1:2, w/w/w), with 40% moisture. They were grown in a climate-controlled cabinet under the conditions previously described. At 4−5 days after emergence, seedlings were transplanted into an outdoor lysimeter, never used for imidacloprid or experiments with radiolabeled compound. Plant spacing was as in the field.

Plants were collected at three stages: B4 (four whole expanded leaves, after 1 month of growth), E4 (star buds, after 2 months of growth), and F (after two-thirds of the florets were blossoming). Leaves and stems were collected separately each as a single sample at the B4 stage or as two samples (bottom and top fractions) at stages E4 and F. E4 flower heads were cut and sampled in two fractions: involucre (bracts and flower head back) and floral dishes with florets.

Before blossoming, flower heads of F plants were placed in a hood in insect-proof paper. During blossoming, pollen was collected by sweeping up with a brush every second day. When two-thirds of the florets were in blossom, flower heads were collected and cut into three fractions: involucre, flower head back, and floret dishes. Seeds already formed were harvested. Samples were weighed, cut into small pieces, and frozen at −80 °C.

Sample Analyses. *Sample Preparation.* Except for pollen, samples were frozen in liquid nitrogen and ground with a ball grinder for 2 min. Ground tissues were homogenized in water/acetonitrile mixture (80:20, v/v) and mixed on a vortex shaker for 1 min. Samples were washed twice with the same mixture. After these washings, <5% of the total radioactivity was again extractable, whatever the washing solvent. Extracts and washing solutions were combined, concentrated to dryness on a rotary evaporator with a bath temperature below 35 °C, and then analyzed by radio-HPLC.

Determination of Total Radioactivity. Radioactivity in tissue samples was measured after combustion of two aliquots (~200 mg FW) (or the total sample of pollen) in an oxidizer (Oxidizer 860, Packard Co., Downers Grove, IL). The resulting $^{14}CO_2$ was trapped in a Carbosorb−Permafluor scintillation mixture (7:10, v/v) (Packard Co.), and the radioactivity was quantified by liquid scintillation counting. To avoid residual contamination from sample to sample, a blank was combusted between each pollen sample.

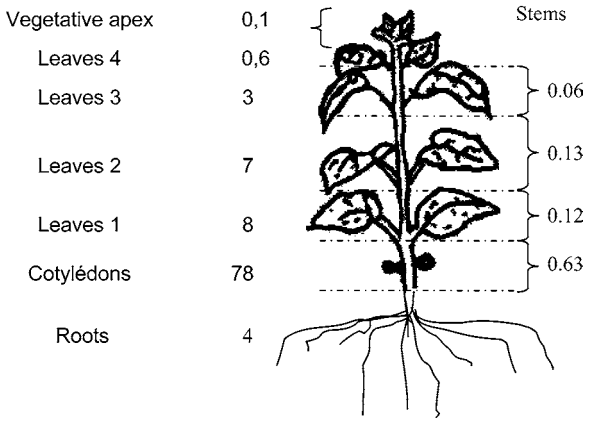

Figure 2. Distribution of radioactivity in plant parts of 1-month-old sunflower seedling (B4 stage) grown from Gaucho-treated seed spiked with [^{14}C]-imidacloprid, expressed as percentage of the radioactivity absorbed by plant.

High-Performance Liquid Chromatography. The extracts were analyzed by reverse-phase HPLC with a Spectra-Physics chromatographic system consisting of a P4000 pump, a Rheodyne 7125 injection valve with a 200 μL injection loop, and a model P1000 Spectra-Physics UV detector set at 272 nm. Separations were carried out on a C-18 Prontosil (6 μm), Bischoff column (250 × 4 mm) with a 1 cm guard cartridge of the same phase at ambient temperature with a flow rate of 1 mL·min^{-1}. The mobile phase consisted of a phosphate buffer, 20 mM, pH 7.0/acetonitrile mixture [95:5, v/v (A) and 50:50, v/v (B)] starting with 100% A with a linear gradient to 100% B at 20 min. The radioactivity of column effluent was monitored on-line with a Flow-one scintillation detector (Packard). Imidacloprid and its metabolites were compared to available standard metabolites on the basis of their retention times. Under our conditions, urea-, guanidine-, hydroxy-, and olefin-imidacloprid and imidacloprid retention times (t_R) were 10.0, 10.8, 11.7, 12.6, and 14.5 min, respectively. 4- and 5-hydroxyimidacloprid were not separated in these conditions.

The amounts of metabolites in radio-HPLC chromatograms were determined as a percentage of the total of all peaks with an area >100 dpm.

Mass Spectrometry Analyses. All experiments were carried out using a quadrupole ion trap mass spectrometer (Finnigan LCQ, Thermo Finnigan, Les Ulis, France) equipped with an electrospray ionization source operated in the positive-ion mode using the following conditions: needle voltage, 5 kV; heated capillary temperature, 220 °C; capillary voltage, 3 V. The analyte solutions in a methanol/water 50:50 (v/v) mixture were directly introduced into the ESI source at a flow rate of 3 μL·min^{-1}.

MS^n experiments were performed on the mass-selected ion in the ion trap mass spectrometer. Ion isolation and collision conditions were optimized separately for each metabolite in order to gain maximal structural information. Helium buffer gas also served as the collision gas for these experiments. All spectra were acquired using automatic gain control.

RESULTS

Uptake and Translocation in the Vegetative Parts of the Plant. Residue distribution was observed in different parts of 1-month-old Gaucho-treated sunflower plants (four pairs of whole-expanded leaves, B4 stage) grown in controlled conditions (**Figure 2**). At this stage, only ~5% of the radioactivity was taken up from the seed dressing. There was a marked

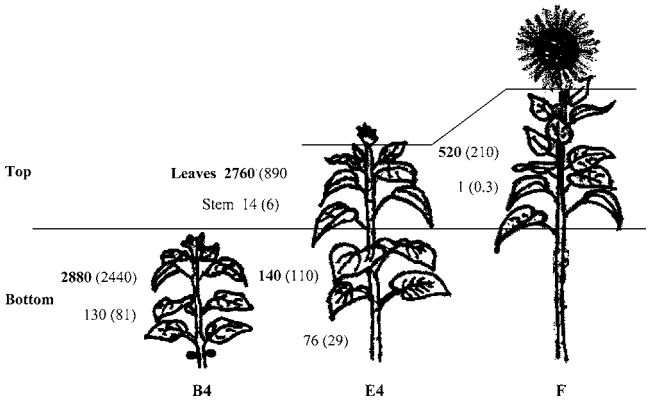

Figure 3. Concentrations in leaves and stems of sunflowers at three growth stages, expressed as imidacloprid or imidacloprid equivalent, ng·g^{-1} (± SD) fresh weight. Boldface numbers indicate leaves and lightface numbers, stem, $n = 5$. nd = not determined.

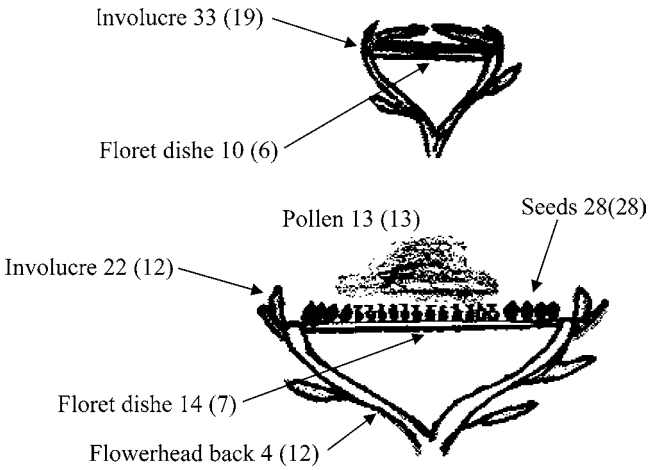

Figure 4. Concentration of imidacloprid residues in various parts of sunflower inflorescences before (E4 stage) and after flowering (F stage), expressed as imidacloprid or imidacloprid equivalent, ng·g^{-1} (± SD) fresh weight, $n = 5$.

decrease in radioactivity from cotyledons to apex. Residues were 50 times more concentrated in cotyledons than in the first leaves and 800 times more than in the apex. Concentrations were reduced by half from each pair of leaves to the next pair up. In the first leaf pair, the concentration of radioactivity was equivalent to 5.8 ± 0.05 μg·g^{-1} FW of imidacloprid, whereas in the fourth leaves (the last totally expanded leaves), the concentration was only 0.57 ± 0.005 μg·g^{-1} FW. High concentrations in cotyledons could be favored by direct cuticle absorption from the soil during seedling emergence. Stem concentrations also followed a gradient, but concentrations (from 88 to 505 ng·g^{-1} FW) were 5−24 times lower than in leaves from the same stratum. In roots, the concentrations (0.56 ± 0.27 μg·g^{-1} FW) were equivalent to concentrations in the first stem section.

In B4 plants (1 month) grown in an outdoor lysimeter, imidacloprid uptake varied from 3 to 10% of the imidacloprid dressing, that is, similar to uptake under controlled conditions. Mean concentrations in leaves were 1% of concentrations in the cotyledons (**Figure 3**). One month later (E4 stage), concentrations in the same leaves (bottom leaves, **Figure 3**) were similar. After full expansion of leaves, accumulation of imidacloprid residues appeared to be negligible in comparison with the previous accumulation. In the more recently developed leaves (top leaves, **Figure 3**), concentrations accounted for only 5% of the burden of bottom leaves. As noted previously, concentrations decreased progressively with growth. It should be noted that in contrast to cabinet experiments, there was considerable variability in concentrations among plants.

At the flowering stage (F), senescent bottom leaves were not analyzed. Mean concentrations in top leaves were 3.5 times higher than in top leaves from the previous E4 stage (**Figure 3**). This difference was not statistically significant; however, these data were in opposition to the gradient of imidacloprid observed in previous stages, which would be expected to increase during further development of F uppermost leaves between both E and F stages.

Translocation toward the Inflorescence. At the E4 stage, concentrations in the green parts of the inflorescence, the involucre (**Figure 4**), were 4 times lower than concentrations in the top leaves. In floret dishes, which anatomically correspond to a modified petiole, concentrations were one-third that of involucre, that is, similar to the top stem, a closely related tissue. On F samples, the involucre of the inflorescence was separated into bracts and inflorescence back. Concentrations in the bracts were 30% lower than in the previous stage, and the inflorescence back was the least contaminated tissue (**Figure 4**). Floret dish concentrations were stable. Thus, no increase in concentrations of radioactivity was observed in inflorescences from E4 stage to F stage.

In pollen, the mean concentration was similar to that of the floret dish. In addition, the mean concentrations in pollen displayed a broad range of values, from a level lower than the limit of quantification (0.5 ng·g^{-1}) to 36 ng·g^{-1}.

Metabolism. In roots, parent imidacloprid was the only compound found. In shoot samples, imidacloprid was always the main compound, and the three major metabolites were identified by HPLC cochromatography of standards. Structures of metabolites were confirmed by mass spectrometry (**Figure 5**). MS/MS experiments were carried out on both chlorine-containing isotopic ions (**Table 1**). Their MS/MS mass spectra showed characteristic fragment ions similar to those of standard compounds.

At all stages, hydroxy- and olefin-imidacloporid were the major metabolites. In leaves of B4 stage (**Figure 6A**), hydroxy- and olefin-imidacloprid were the sole metabolites, accounting for only ∼4% of the total leaf radioactivity. By contrast, in cotyledons (**Figure 6B**), one-third of the radioactivity was present as metabolites.

Together with both of these metabolites, the guanidine residue was detected in low quantity (∼15% of metabolite radioactivity). Sometimes a minor peak (<2% of the radioactivity) occurred; although not formally identified by mass spectrometry, it chromatographed as the urea-imidacloprid, that is, the deaminated guanidine residue. At both other stages, metabolites accounted for 30−50% of radioactivity in leaf samples, without significant differences between the stages. **Figure 7A** shows a typical radiochromatogram of leaf extract, but the percentages of metabolites varied in accordance with the plant. The suspected urea metabolite and guanidine-imidacloprid were not always detected. As in leaves, radioactivity in stem extracts (**Figure 7B**) was mainly imidacloprid with 25% of the radioactivity as metabolites.

Two further minor polar peaks were detected at $t_R = 4.1$ and 8.1 min. They did not correspond to known metabolites and were not identified. At F stage the metabolite pattern was determined only in pollen. Owing to the low amount of radioactivity together with only a small quantity of pollen

Table 1. Data of MS/MS Fragmentation of the Three Major Metabolites with 35 or 37 Chlorine Isotope

name	Cl isotope	molecular mass	quasi-molecular ion $[M + H]^+$, *m/z*	characteristic fragment ions, *m/z*
guanidine analogue	35	210	211	194, 175, 126, 84
	37	212	213	196, 175, 128, 84
4- or 5-hydroxyimidacloprid	35	271	272	254, 228, 226, 225, 191, 190
	37	273	274	256, 230, 228, 227, 191, 190
olefin-imidacloprid	35	253	254	236, 208, 207
	37	255	256	238, 210, 209

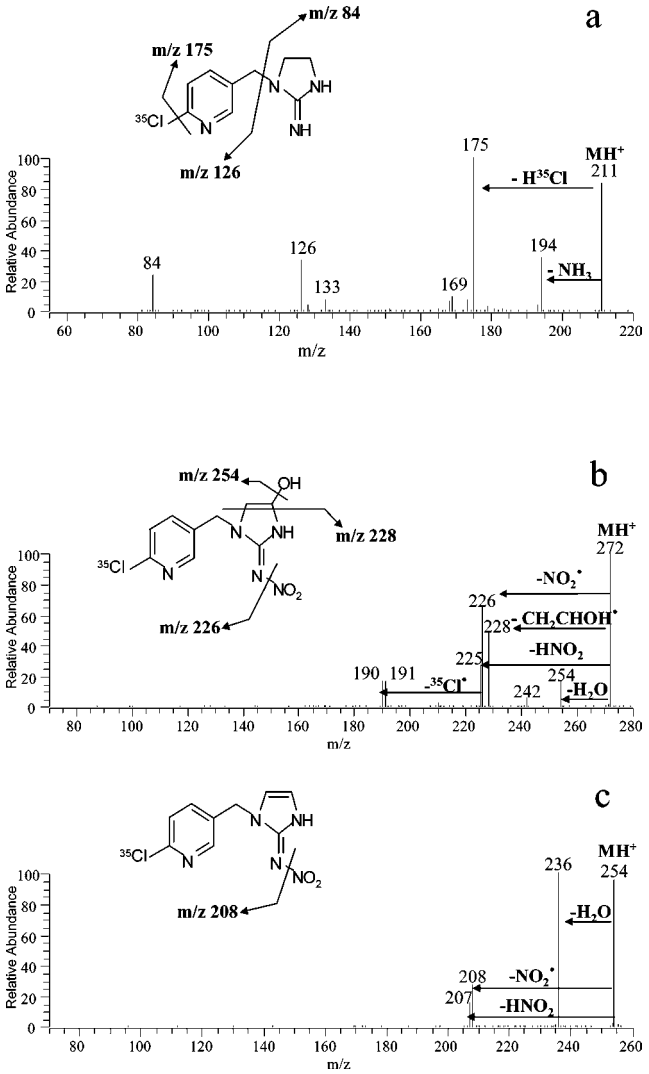

Figure 5. Mass spectrum of the different metabolites of imidacloprid from leaf extracts: (**a**) guanidine; (**b**) 4-hydroxyimidacloprid; (**c**) olefin. MS spectra were of quasi-molecular ^{35}Cl isotopic ions. Metabolite formulas are inset with a diagram showing the locations of fragmentations.

samples, pollen extracts were analyzed by HPLC as a single sample. A single peak corresponding to imidacloprid was detected.

DISCUSSION

The gradient of the distribution of the radioactivity in sunflowers showed that the accumulation of imidacloprid residues was a function of time and was therefore correlated with leaf age. This showed that imidacloprid residues predominantly moved in the upward direction from roots. It was consistent with its log $K_{ow} = 0.57$. Indeed, Bromilow and Chamberlain (*15*) showed that the molecules with such a log

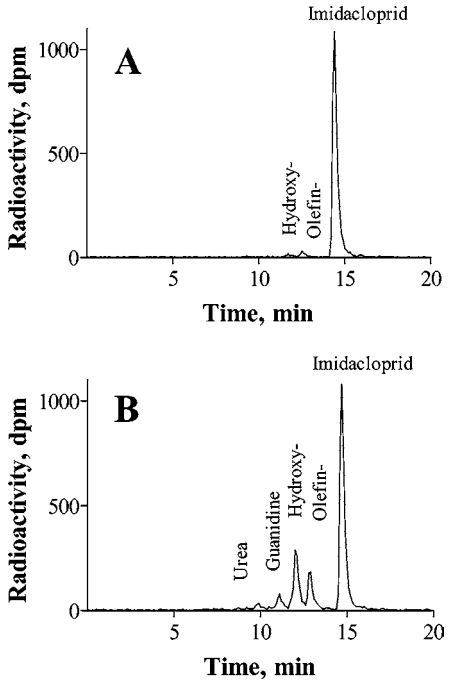

Figure 6. Radiochromatograms of an extract of leaves (**A**) and cotyledons (**B**) of sunflowers 1 month after seed treatment (stage B4) by Gaucho plus [^{14}C]imidacloprid.

K_{ow} were xylem-mobile substances, transported only by the transpiration stream. Moreover, in soil, the difference between the distribution of the roots system and the diffusion of imidacloprid emphasized this acropetal gradient. Despite the high water solubility of imidacloprid (0.610 g·L^{-1}), the leaching of imidacloprid in soil is limited, the molecule being confined to the top 30 cm (*16*−*18*). The volume of soil contaminated by imidacloprid is expected to be restricted to close to the treated seed. In contrast, sunflower roots spread downward to a depth of ∼1.5 m (*19*), and the apex roots (which are the most effective for absorption) will grow beyond the contaminated zone. However, the morphology of sunflower roots and their distribution in the soil is twofold, with a shallow fibrous root mass (fascicular roots), which grows horizontally in the superficial layer of the soil, together with a deeper root system. The preferential development of one or the other system can be influenced by different soil structures or by climatic conditions during growth. Fascicular roots grow in the soil with higher imidacloprid concentrations and could absorb imidacloprid continuously. As was indicated by the low uptake of radioactivity in our experiments and in other trials (*10*), the quantity that remained in the soil at blossoming stage was estimated to be ∼90% of radioactivity deposited on the seed coating. This formed a huge pool of the compound in the soil, most likely in the form of imidacloprid. In 3-month-old Gaucho-treated sugarbeets, Westwood et al. (*20*) showed that almost all extracted residues still present in the soil were imidacloprid.

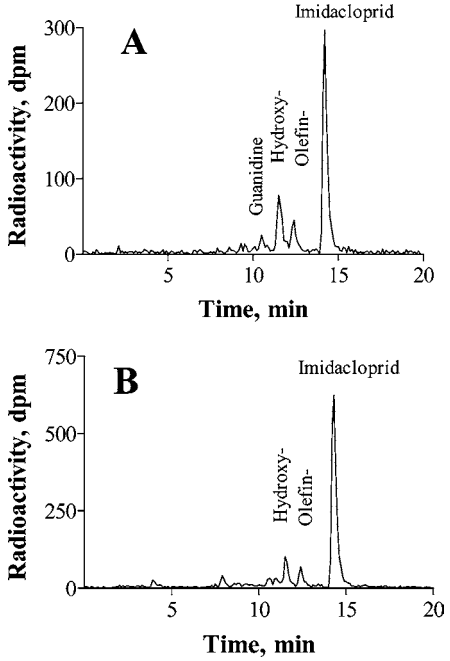

Figure 7. Typical radiochromatograms of an extract of leaves (**A**) and stems (**B**) of sunflowers, 3 months after seed treatment (stage F) by Gaucho [^{14}C]imidacloprid.

Half-lives of imidacloprid in cropped soil were estimated to be >45 days (*21*, *22*). Moreover, Bonmatin et al. (*11*) showed that sunflowers grown from untreated seeds absorbed imidacloprid from a soil in which an imidacloprid-treated crop had been grown the previous year. Therefore, during the growth period of sunflowers (~70 days), imidacloprid would still be available for plant uptake at blossoming stage. The balance of uptake between the two types of root system and the imidacloprid distribution in the soil would be a function of the growing conditions and could explain the difference in concentrations found in pollen in the different trials. This is all the more likely because the amount of water available in the soil could favor water uptake, and consequently imidacloprid uptake, by one or the other root system at a given time. In our experiments, imidacloprid concentrations in pollen were higher than those reported in other studies (*10*−*12*). However, in our trials, Gaucho-treated seeds were dressed with 1 mg of imidacloprid, that is, 30% higher than the recommended application rate for sunflower crops. At equivalent doses, our concentrations would be expected to be in the same range of values determined by Bonmatin et al. (*11*) in field crop pollen. In contrast, our values would be expected to be ~2 times higher than those reported by Schmuck et al. (*10*) in a study performed, like ours, with labeled imidacloprid or those with non-radiolabeled imidacloprid reported by Lagarde (*12*) from field samples of pollen, which found average concentrations ranging from 2 to 8 ppb.

Differences in imidacloprid uptake during the blossoming period could be the cause of differences in concentrations in pollen. Moreover, fascicular roots could reach the contaminated zone of neighboring plants both in field conditions and in a lysimeter. Conversely, fascicular root expansion in an isolated pot experiment would prevent this phenomenon from occurring and the rate of contamination of pollen would thus be lower, as reported by Schmuck et al. (*10*).

Similar processes could explain the relatively unexpected high concentration in top leaves at the same period. Bonmatin et al. (*11*) reported similar observations on Gaucho-treated sunflowers grown in field conditions, particularly with the Albena cultivar. To explain these data, these authors suggest a remobilization process: imidacloprid or its residues, accumulated in bottom leaves during the vegetative stage, could be transferred toward the upper part of plants during the reproductive stage (blossoming or seed formation).

The high variability of our data compared to other studies could also be due to the outdoor conditions. We collected pollen, inflorescence by inflorescence, like Schmuck et al. (*10*), but our study was performed under outdoor conditions rather than with sunflowers growing under optimized climatic conditions. Sampling methods are likely also a determining factor in variability. In field trials performed by Bonmatin et al. (*11*) and Lagarde (*12*), pollen was collected from several inflorescences to obtain sufficient quantities (several grams) for analysis; this sampling method tends to smooth variability in inflorescences.

The majority of imidacloprid residues in sunflowers were the parent compound, which was metabolized by two pathways, as observed in other plants (*13*). The denitrification pathway resulted in a very low quantity of the final urea metabolite, although the intermediary product (imidacloprid-guanidine) sometimes represented up to 20% of the present metabolites. The second pathway resulted from the imidazolidine oxidation. The latter led to the formation of 4- or 5-hydroxyimidacloprid and subsequently to olefinic residues. The degradation of imidacloprid in soil gave the same metabolites (*21*, *22*). However, the plant uptakes of these soil metabolites seemed low; indeed, no metabolites were detected in roots. Moreover, sunflowers metabolized imidacloprid in these residues as shown on semisterile culture or on excised leaf experiments (results not shown). Our studies did not permit us to determine if the presence of the hydroxy and olefinic residues in stem resulted from in situ metabolism or translocation from leaves. Distinguishing between these two processes might be of consequence for the risk assessment. Indeed, both compounds had insecticide activity (*14*, *23*) and consequently may be partially responsible for the sublethal syndrome observed in bees. Any translocation from leaves would likely be via the phloem, which should also lead to contamination of nectar, which is made from phloem-transported products. The concentration in nectar was not determined here, but imidacloprid alone was detected in nectar by Schmuck et al. (*10*). In pollen, similar results were obtained in both studies.

In some plants, a minor pathway of imidacloprid metabolism is the chloronicotinic acid pathway resulting from the cleavage of the methylene bridge (*13*). Our studies using [^{14}C-*imidazolidine*]imidacloprid would not have led to ^{14}C-labeled chloronicotinic acid metabolites. Nevertheless, the peaks at 4.1 and 8.1 min on HPLC with UV detection did not correspond to any described metabolites of imidacloprid. It may be an imidazoline ring residue, resulting from methylene bridge hydrolysis. In sunflowers, the low amount of this compound should mean that this pathway was of minor incidence.

Radioactivity concentrations in pollen appeared to vary significantly depending on the study. A number of factors could explain these variations: first, the conditions of the tests, from strictly controlled to open fields; second, the infinitesimal quantities of imidacloprid translocated to the pollen, which were estimated at ~0.005% of total quantities absorbed by plants. Thus, slight variations of the uptake of imidacloprid during blossoming could have strong effects on the concentration in pollen. Finally, the sampling method of pollen could fit the interindividual differences.

The difficulty in assessing the risk of bee exposure to imidacloprid, and therefore in determining if Gaucho treatment

was really responsible of the bee syndrome, was (i) by contrast to the DL_{50}, to determine the toxic sublethal dose on bees (*24*) [in accordance with the multiplicity of used tests, toxic sublethal concentrations considerably varied, from 2 to 60 ppb (*25*−*27*)]; (ii) to determine the amount of imidacloprid that could really contaminate bees during pollen or nectar foraging (is it the quantity of pollen cropped by bees or only the pollen consumed that should be considered to calculate the exposure amount?); and (iii) to link the concentration in pollen or nectar and a sublethal dose. In this context, even if variations in imidacloprid residue concentrations in pollen were observed, the range was relatively tight when compared with the wide distribution of the different data in the literature concerning sublethal doses. Thus, pollen content may be a relevant value for imidacloprid exposure evaluation (*28*), although it only represents a very small part of original plant contamination.

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