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PAPER

Rapid analysis of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds

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Regarding the hypothesis that neonicotinoid insecticides used for seed coating of agricultural crops – mainly corn, sunflower and seed rape – are related to the extensive death of honey bees, the phenomenon of corn seedling guttation has been recently considered as a possible route of exposure of bees to these systemic insecticides. In the present study, guttation drops of corn plants obtained from commercial seeds coated with thiamethoxam, clothianidin, imidacloprid and fipronil have been analyzed by an optimized fast UHPLC-DAD procedure showing excellent detection limits and accuracy, both adequate for the purpose. The young plants grown both in pots – in greenhouse – and in open field from coated seeds, produced guttation solutions containing high levels of the neonicotinoid insecticides (up to 346 mg L^{-1} for imidacloprid, 102 mg L^{-1} for clothianidin and 146 mg L^{-1} for thiamethoxam). These concentration levels may represent lethal doses for bees that use guttation drops as a source of water. The neonicotinoid concentrations in guttation drops progressively decrease during the first 10–15 days after the emergence of the plant from the soil. Otherwise fipronil, which is a non-systemic phenylpyrazole insecticide, was never detected into guttation drops. Current results confirm that the physiological fluids of the corn plant can effectively transfer neonicotinoid insecticides from the seed onto the surface of the leaves, where guttation drops may expose bees and other insects to elevated doses of neurotoxic insecticides.

Introduction

Honey bee colony losses are a complex phenomenon often characterized by a rapid disappearance of honey bee colonies failing to return to their hive, and the presence of capped brood with a live queen bee and of food stores in the hive, called Colony Collapse Disorder (CCD) syndrome.[1,2] This phenomenon has been observed worldwide in the last few years,[3–5] with a rapidly increasing number of cases in Europe,[6] USA[7] and Japan.[3] For instance, over winter 2007–2008, 36% (2.4 million) of America's bee hives were lost.[8] European figures follow the same trend,[6] with peaks of up to 60% of the hives. This honey bee crisis and the consequent reduction in the pollination of flowering plants, induces adverse effects on beekeeping, agriculture and natural ecosystems, and it actually constitutes a worldwide emergency both from an economic and an ecological standpoint.

Many hypotheses, such as infections of parasitic mites,[9] viruses,[10] chronic exposure to sub-lethal doses of insecticides[11–14] or acute effects of neonicotinoid insecticides[15] were formulated to account for bee decline. Up to the present none of them have been confirmed or refuted and their impact has never been clearly quantified, so that a multifactorial origin of colony losses is often suggested in the qualified literature.[3] Moreover, first reports of the surveillance networks on bee decline[6–8] seem to indicate

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Environmental impact

The significant contamination of the guttation drops produced by young corn plants grown from seeds coated with neonicotinoid insecticides may represent a risk for honey bees and other insects. With the aim to assess this possible exposure route for bees, starting from quantitative data, a simple and rapid analytical method for the accurate determination of neonicotinoid insecticides in guttation solutions has been optimized and then applied to different series of real samples collected both in the laboratory and in the field. The optimized procedure could be a very useful tool for the future exposure studies and the consequent risk assessment for honey bees.

a high temporal and geographical variability in colony losses. In southern Europe significant peak events – different from the winter colony losses – were detected at the beginning of spring.[16,17,6] This supports the hypothesis that they were related to the acute toxic effects of neonicotinoid insecticides released in the environment by agricultural practices, in particular during corn sowing.[16,18] It is worthwhile to notice that in Italy the use of corn seed coated with neonicotinoids was banned in September 2008 and no cases of colony collapse were recorded in the springs of 2009 and 2010.[19,20]

Actually, neonicotinoid insecticides are widely used in agriculture and the seed coating is used all over the world to ensure a broad range pest control in several crops, including corn (*Zea mays* L.).[18] Neonicotinoids are water soluble compounds and systemically translocate to plant tissues protecting young plants from root-eating insects and, after emergence, also from sucking insects – such as leafhoppers and aphids – responsible for the transmission of plant viruses.[18] Nevertheless, the neonicotinoids hypothesis of bee decline runs counter to the experimental observation that the amounts of neonicotinoids detected in nectar or pollen (or dew) of the plants were always lower than 10 ppb,[21] while higher concentrations (>40 ppb) are necessary for abnormal honeybee foraging behaviour or bee loss (>0.5 ppm).[12] Although this prompted investigations into other mechanisms of toxicity for bees, such as the possible effects of sub-lethal doses of insecticides on the course of common bee pathologies, studies on the real ways in which bees are exposed to neonicotinoid insecticides seem to have lacked in quantitative data, so far.

Recently, a novel way of possible exposure (and intoxication) of honey bees to neonicotinoid insecticides was proposed by Girolami and co-workers,[22] who postulated and evidenced the translocation of a significant amount of toxic neonicotinoid insecticide from the coated seed to the guttation drops of young corn plants. Guttation is a physiological phenomenon (often confused with dew) characterized by the exudation of drops of xylem sap through the hydathodes, the porous tissues present at the leaf tops and margins, as an effect of roots pressure.[23–25] In corn crops, drinkable guttation solutions can persist into the crown cup of the young plants for the whole day.

In this work, the effective contamination of the guttation drops obtained from young corn plants grown from seeds coated with neonicotinoids has been studied. With the aim to assess this possible exposure route for bees, starting from quantitative data,[26] a fast liquid chromatographic procedure for the rapid, sensitive and accurate analysis of neonicotinoids in guttation drops has been optimized and then applied to different series of guttation solutions collected both in the laboratory and in the field.

Experimental section

Corn seedlings were obtained from seeds (hybrid PR34N84, Pioneer Hi-Bred Italy) commercially available in 2008, 2009 and 2010 and coated with neonicotinoid insecticides: imidacloprid (*N*-[1-[(6-chloro-3-pyridyl)methyl]-4,5-dihydroimidazol-2-yl] nitramide; Gaucho®, Bayer Cropscience, 0.5, 1 or 1.25 mg/seed); clothianidin ((*E*)-1-(2-chloro-1,3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine; Poncho®, Bayer Cropscience, 1.25 mg/seed); thiamethoxam (3-[(2-chloro-1,3-thiazol-5-yl)methyl]-5-methyl-*N*-nitro-1,3,5-oxadiazinan-4-imine; Cruiser®, Syngenta International, 0.6 or 1 mg/seed). Seeds coated with fipronil (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-(trifluoromethylsulfinyl)-1*H*-pyrazole-3-carbonitrile; Regent®, BASF SE, 0.5, 0.75 or 1 mg/seed), a non-systemic N-phenylpyrazole insecticide, were also utilized. Untreated seeds (hybrid PR33A46, Pioneer Hi-Bred Italy) or seeds coated with fludioxonil and metalxyl-M (Celest®, Syngenta International, 2.4% and 0.93%, respectively) fungicides were used as controls.

Corn seedlings were grown both in open field (April 2009 and 2010, seeds spaced 20 cm within the row and 75 cm between rows by using a Monosem NG Plus pneumatic drilling machine) and in the laboratory (greenhouse, November 2008–October 2010) with seeds sown in pots (15 cm in diameter) and growing 2–5 plants per pot. A total of 6–8 pots for each insecticide were used and equal numbers of pots were sown with control seeds (uncoated or coated with fungicides).

For the first 20 days after the emergence of the seedlings, guttation drops were collected every morning by a pipette from the leaves of corn plants (from single plants or homogeneous groups of plants). Samples were stored at 4 °C until the instrumental analysis.

For analytical determinations, a new, fast liquid chromatographic (ultra high performance liquid chromatography, UHPLC) procedure was optimized on a Shimadzu Prominence UFLC-XR chromatograph equipped with a Shimadzu SIL 20AC-XR auto sampler, Shimadzu SPD-M20A UV-Vis diode array detector and a Shimadzu XR-ODS II (2.2 μm, 2 × 100 mm) analytical column with a Phenomenex security guard –Phenomenex ODS (4 × 2.0 mm) precolumn. The following instrumental parameters were adopted: eluent flow rate of 0.4 mL min^{-1}, gradient elution (0–1 min: 77/23% water–acetonitrile; 1–2.2 min, linear gradient to 100% acetonitrile; 2.2–3.5 min, 100% acetonitrile), 5 μL of injector volume, 45 °C of column temperature. Detector signal at $\lambda = 215$ nm for fipronil, $\lambda = 252$ nm for thiamethoxam and $\lambda = 269$ nm for clothianidin and imidacloprid were adopted for analyte quantification. Although thiacloprid and acetamiprid are not used for corn seed coating, they can also be separated and quantified ($\lambda = 244$ nm) by the optimized analytical method. Instrumental calibration (external) was performed by analysis of standard solutions in the 0.05–10 mg L^{-1} concentration range of analytes in 50% water–methanol. Sample analyses were performed by direct injection of the guttation solutions, after filtration on a Millex HV 0.45 μm (Millipore) syringe filter. Concentrated samples were diluted by addition of a 50% water–methanol solution in the injection vials.

Fipronil, thiamethoxam, clothianidin, imidacloprid, acetamiprid and thiacloprid were purchased from Fluka (Pestanal, purity >99.7% for the five neonicotinoids and >97.5% for fipronil). Methanol (VWR) and acetonitrile (Riedel de Haen) were of HPLC grade and water was purified by a Millipore MilliQ equipment.

Results and discussion

UHPLC analytical procedure

Trace analysis of neonicotinoid insecticides in environmental matrices is currently performed by conventional reverse phase

liquid chromatographic procedures using different detection strategies.[27–32] Even though HPLC-DAD methods are less sensitive and selective with respect to procedures using mass-spectrometric or electro-chemical detectors, our preliminary analysis of guttation drops[22] showed that very high concentration levels of insecticide could be effectively present in these samples. Therefore, the analytical drawbacks typical of ultra-traces environmental analysis (*i.e.* lack of sensitivity or selectivity in the real samples) could be a minor problem in this case. In other words, the use of a dedicated instrumentation (UHPLC with high efficiency C18 column, 2.2 μm particles) can reduce the analysis time while maintaining high analytical performances, both in terms of sensitivity and selectivity.

Actually, the optimized fast procedure reduces analysis time to 5 min (Fig. 1) and no chromatographic interferences have been observed in the detection of the six insecticides in real samples. Precision levels of 0.2% for thiamethoxam, 0.3% for clothianidin and imidacloprid have been computed from replicate analysis of real samples (conc > 2 mg L^{-1}) and 0.8% for fipronil from replicate analyses of standard solutions. The developed method reaches instrumental detection limits of 4.5 μg L^{-1} for thiamethoxam and thiacloprid, 5.1 μg L^{-1} for clothianidin and fipronil, 4.8 μg L^{-1} for imidacloprid, and 5.4 μg L^{-1} for acetamiprid, all evaluated using the procedure suggested by IUPAC.[33,34] This means that quantification limits for the analysis of real samples, evaluated as LOQ = 10 × LOD/3,[34] are 15 μg L^{-1} for thiamethoxam and thiacloprid, 17 μg L^{-1} for clothianidin and fipronil, 16 μg L^{-1} for imidacloprid and 18 μg L^{-1} for acetamiprid.

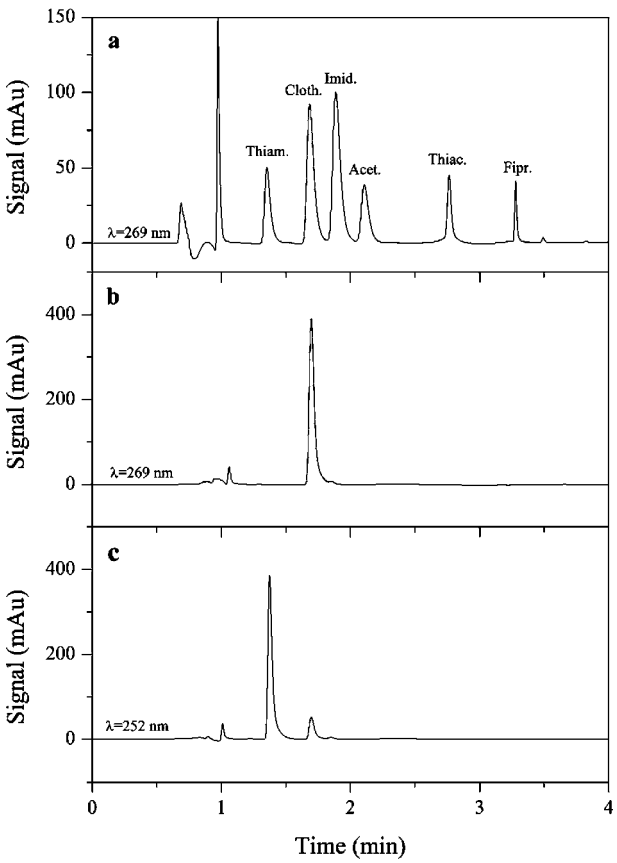

Fig. 1 Chromatograms of (**a**) 10 mg L^{-1} standard solution of the six insecticides, (**b**) guttation sample collected from corn plants treated with Poncho® (clothianidin, 42 mg L^{-1}) and (**c**) guttation sample collected from corn plants treated with Cruiser® (thiamethoxam, 43 mg L^{-1}).

The linearity range of instrumental responses was tested with up to 100 mg L^{-1} concentrations of standard solutions, obtaining a linear calibration function ($r^2 > 0.999$, $p < 10^{-8}$) for each analyte. Spiked samples (guttation solutions from seeds coated with fungicides and added with 0.1–1 mg L^{-1} of thiamethoxam, clothianidin and imidacloprid) showed recovery factors in the range 91–108%. Moreover, the absence of chromatographic interferences for the UHPLC-DAD method was verified by LC-ESI/MS analysis of both spiked and real samples, using identical chromatographic conditions, and obtaining MS signals attributable to the single analyte for each insecticide.

Corn plant guttations

The guttation phenomenon is affected by a number of factors such as humidity, temperature, growth stage, water stress, root depth and soil water potential. Moreover the insecticide residues in guttation fluid exhibit wide variability due both to factors affecting guttation as a phenomenon and to formulation, metabolism within the plant, application methods, adjuvant, solubility of the active ingredient and plant species.[26] Thus, detailed studies need to be conducted to better understand guttation as a possible exposure route to neonicotinoids for honey bees. In this respect, the fast analytical methods described in this paper could turn out to be very useful. Some applications of the proposed procedure are here presented and discussed.

In a first campaign (November 2008) corn plants were grown in pots in greenhouse. The guttation drops collected were divided into six periods in order to obtain enough sample to perform both an UHPLC analysis and toxicological tests.[22] The results of instrumental analysis revealed the effective translocation of the insecticides from the seeds to the leaves of the plants in the whole period when guttation occurs, *i.e.* 15–20 days after the seedling emergence and with a production of about 30–150 μL/day/plant of water. The concentrations of the insecticides in the guttation drops were surprisingly high for all the three neonicotinoids while for fipronil, a non-systemic phenylpyrazole insecticide, they were always below the detection limit (LOD = 5.1 μg L^{-1}). Guttation solutions from control seedlings (obtained both in laboratory and in the field from non-coated seeds or from seeds coated with fungicides) contained no detectable concentration of insecticides (*e.g.*, below the instrumental detection limits: 4.5 μg L^{-1} for thiamethoxam, 5.1 μg L^{-1} for clothianidin and 4.8 μg L^{-1} for imidacloprid).

Insecticide concentrations showed a characteristic temporal variation: concentration rapidly decreased during the first 10 days after the seedling emergence (Fig. 2) while it increased again, in the reported experimentation, during the last 10 days of the guttation phenomenon, when it is considerably reduced and water evaporation may significantly concentrate the solute. Thiamethoxam (Cruiser® 1 mg/seed) observed concentration decreased from 24.29 mg L^{-1} during the 1st day after the seedling emergence to 3.55 mg L^{-1} for the 8th–10th days and it increased again to 8.32 mg L^{-1} during the subsequent 10 days. Clothianidin (Poncho® 1.25 mg/seed) concentration ranged from 35.99 mg L^{-1} during the 1st day after the seedling emergence to 8.82 mg L^{-1} for

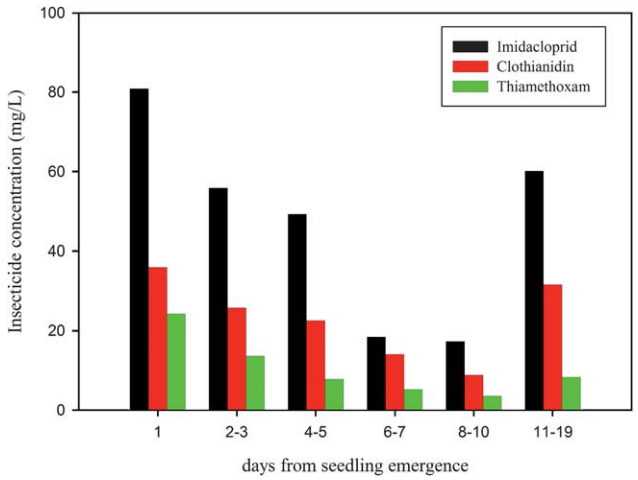

Fig. 2 Concentration of neonicotinoid insecticides in guttation drops of corn seedlings obtained from coated seeds (greenhouse).

the 8th–10th days and it increased again to 31.64 mg L^{-1} during the last 10 days. Imidacloprid (Gaucho® 0.5 mg/seed) concentration ranged from 80.87 mg L^{-1} during the 1st day after the seedling emergence to 17.30 mg L^{-1} for the 8th–10th days and it increased again to 60.13 mg L^{-1} during the last 10 days.

Although successive sowing experiments (greenhouse, spring–summer 2009) have always confirmed this temporal trend, a high variability in the translocation efficiency of each insecticide has been observed (concentrations range up to 346 mg L^{-1} for imidacloprid, 102 mg L^{-1} for clothianidin and 146 mg L^{-1} for thiamethoxam). It is worthwhile to note that, from our results, the insecticide concentrations in guttation drops seem to be only partially related to the original amount in the seed: for instance, the growing of single seedlings per pot produced guttation drops, in the first six days from the emergence, containing decreasing concentrations of imidacloprid, in the range 115–25 mg L^{-1} from seed treated with 1.25 mg/seed and 110–64 mg L^{-1} from seed treated with 0.5 mg/seed. This seems to support the hypothesis that both environmental and physiological conditions (*i.e.* soil temperature and moisture, air humidity) mainly affect the translocation efficiency and the actual concentration of the insecticides in guttation drops.[26]

In this connection we observed that for seedlings grown under dry conditions (both soil and air), guttations appeared later and a lower volume of water was produced. On the other hand, under wet conditions the washing-out of the insecticides from the soil is particularly effective for thiamethoxam which is the most water-soluble neonicotinoid. In a trial conducted in experimental parcels (greenhouse, November 2009) using usual soil with three different levels of moisture (obtained by different water supplies), we observed concentrations of thiamethoxam in guttation drops in the range 14–155 mg L^{-1} in plants grown under wet conditions (the water content in the soil was near saturation),† 27–253 mg L^{-1} with moderate soil humidity (the parcel had a water content

† It is possible to define as saturated a soil with all pores filled with water. After 24–48 h, when free drainage occurs, the soil reaches the field capacity (FC). When the plants have extracted all water present in the soil they can, the permanent wilting point (PWP) condition is obtained.[35]

Table 1 Concentration ranges of neonicotinoid insecticides in guttation drops collected at the top and at the crown cup of the corn leaves during the first six days after the emergence of the corn seedlings

		Insecticide concentration (mg L^{-1}) in guttation drops	
Corn seed	Active principle	At the top of the leaves	At the crown cup
Gaucho®, 1.25 mg/seed	Imidacloprid	345.8–102.9	120.4–8.2
Poncho®, 1.25 mg/seed	Clothianidin	101.7–76.2	47.0–7.3
Cruiser®, 1 mg/seed	Thiamethoxam	40.8–16.2	25.5–2.9

approximately close to the field capacity (FC)) and 34–1154 mg L^{-1} under dry conditions (the parcel had a water content slightly above the wilting point (PWP)).

The comparison between guttation drops collected from the top and from the crown cup of the leaves evidenced that significantly lower concentrations of the insecticides are present in the latter (Table 1). This is probably due to the dilution of guttations by dew or to degradation processes of the insecticides, for example photodegradation.

In open field cultivation, both the high contents of neonicotinoids in guttation drops and the characteristic exponential decay of the concentration during the first 10 days after the emergence were confirmed,[26,36] but with higher concentration variability than that observed in greenhouse. For instance, the parallel field cultivation (April 2010) of different coated seeds produced guttation drops with concentration peaks (1st day after the seedling emergence) in the range 77–222 mg L^{-1} for imidacloprid, 19–46 mg L^{-1} for clothianidin and 79–227 mg L^{-1} for thiamethoxam.

We also observed that guttation samples often contain traces of other neonicotinoids than the seed coating insecticide. This is possibly attributable to a contamination effect during the coating procedure, as confirmed by an analysis of the original seeds, during which we found 30 μg/seed of thiamethoxam in 2008 Gaucho® seeds (1.25 mg/seed of imidacloprid). Nevertheless, all guttations from plants grown from Cruiser coated seeds (thiamethoxam) contain correlated concentrations of clothianidin (*ca.* 10% with respect to the coating insecticide, Fig. 1c) which is a well-known degradation product of thiamethoxam.[37]

As for the toxic effects of these guttation solutions – if orally administered to honeybees – they induce two characteristics neurotoxic symptoms, *i.e.* abdomen contraction and irreversible wing block. The time scale is of a few minutes and the concentration of the neonicotinoid insecticides was so high that all the honeybees tested died in up to fifteen minutes.[22] As the time scale is so short, guttation drops could explain the sudden disappearance of worker bees during the early spring if they use corn guttations for their foraging. Literature[23] and direct beekeepers' observations report that guttation drops can be used by honeybees for their foraging especially in the early spring when they require intensive drinking activity and water-fetching for the hive.[24] However, honeybees are likely to use guttation for their foraging in particular conditions of drought when no other major visible sources of water are present thereabout.

Conclusions

A fast UHPLC-DAD analytical procedure has been optimized for the rapid determination of neonicotinoid insecticides in guttation drops. The method reduces the analysis time to 5 min and shows adequate sensitivity, selectivity and excellent repeatability and detection limits for the intended purpose. The method has been successfully applied to the analysis of real samples obtained from corn seedlings grown both in greenhouse and in open field, confirming the effective translocation of neonicotinoids from coated seeds to seedling guttations. These solutions may represent a possible route of exposure to lethal doses of the insecticides for bees and other insects.

Because guttation is affected by several factors that cause a high variability both in its intensity and in the insecticide content, further experiments are needed to better understand the phenomenon and the consequent risk assessment for honey bees. The fast analytical procedure described could be a very useful tool for more accurate exposure studies. In any case, the presence of a source of water carrying neonicotinoid concentrations in solution up to the levels shown in the current study, and persisting for weeks on more than a million hectares in northern Italy alone, is a threatening scenario that seems to be incompatible with ecologically acceptable conditions.

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