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ORIGINAL PAPER

# UHPLC-DAD method for the determination of neonicotinoid insecticides in single bees and its relevance in honeybee colony loss investigations

**Andrea Tapparo · Chiara Giorio · Lidia Soldà · Sara Bogialli · Daniele Marton · Matteo Marzaro · Vincenzo Girolami**

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**Abstract** In the understanding of colony loss phenomena, a worldwide crisis of honeybee colonies which has serious consequences for both apiculture and bee-pollination-dependent farm production, analytical chemistry can play an important role. For instance, rapid and accurate analytical procedures are currently required to better assess the effects of neonicotinoid insecticides on honeybee health. Since their introduction in agriculture, neonicotinoid insecticides have been blamed for being highly toxic to honeybees, possibly at the nanogram per bee level or lower. As a consequence, most of the analytical methods recently optimized have focused on the analysis of ultratraces of neonicotinoids using liquid chromatography–mass spectrometry techniques to study the effects of sublethal doses. However, recent evidences on two novel routes—seedling guttations and seed coating particulate, both associated with corn crops —that may expose honeybees to huge amounts of neonicotinoids in the field, with instantly lethal effects, suggest that selected procedures need optimizing. In the present work, a simplified ultra-high-performance liquid chromatography–diode-array detection method for the determination of neonicotinoids in single bees has been optimized and validated. The method ensures good selectivity, good accuracy, and adequate detection limits, which make it suitable for the purpose, while maintaining its ability to evaluate exposure variability of individual bees. It has been successfully applied to the analysis of bees in free flight over an experimental sowing field, with the bees therefore being exposed to seed coating particulate released by the pneumatic drilling machine.

**Keywords** Honeybees · Neonicotinoid insecticides · Liquid chromatography · Quick, easy, cheap, effective, rugged, and safe extraction · Mass spectrometry

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A. Tapparo (✉) · C. Giorio · L. Soldà · S. Bogialli · D. Marton
Dipartimento di Scienze Chimiche, Università degli Studi di Padova,
via Marzolo 1,
35131 Padova, Italy
e-mail: andrea.tapparo@unipd.it

M. Marzaro · V. Girolami
Dipartimento di Agronomia Animali Alimenti Risorse Naturali e Ambiente, Università degli Studi di Padova,
Agripolis – viale dell'Università 16,
35020 Legnaro, Padova, Italy

V. Girolami
e-mail: vincenzo.girolami@unipd.it

## Introduction

A recent, invasive syndrome affecting honeybee (*Apis mellifera* L.) colonies in the Northern hemisphere, named colony collapse disorder, is characterized by a sudden, massive disappearance of honeybees from the hive [1–5]. Although several causes have been hypothesized, pesticides have received more consideration by the scientific community. The experimental evidence for an association between the colony loss phenomena, including losses occurring in early spring, and the use of neonicotinoid insecticides, in particular as seed dressing in corn crops, an agricultural practice used worldwide, is extensive and there is sufficient mechanistic understanding to put the question of causality beyond reasonable doubt [6–13].

Although spring mortality is characterized by a rapid disappearance of bee colonies (a typical short-term effect), scientific efforts were in most cases based on exposure to sublethal doses of neonicotinoids, which may weaken the colonies and make them more susceptible to both common and new diseases [10, 14–19]. In fact, since Greatti et al. [20] demonstrated the possible release of seed coating insecticides through the fan drain of the pneumatic drilling machine during corn sowing and hypothesized that bees are exposed to the neonicotinoid-containing particles falling off to the vegetation at the field margin, experimental results have shown that the neonicotinoid content in nectar and pollen collected from the surrounding vegetation was always around 50 ppb or lower [12, 21–23], whereas higher doses are necessary for an acute toxic effect [13, 16–18, 24].

In this connection, Girolami and coworkers have recently proposed two novel routes of exposure to and intoxication with neonicotinoids which may justify such a sudden spring mortality: the translocation of a significant amount of neonicotinoids from the coated seed to the guttation drops of young corn plants [6, 24] and the direct powdering with neonicotinoid-containing particles of foraging bees in free flight accidentally crossing the sowing fields [11, 21, 25, 26].

Exposure and monitoring studies also promoted several analytical methods [27–31], mainly using high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) [32–35], for the determination of neonicotinoid insecticide content in exposed bees. In these methods, but also in methods for the analysis of simpler matrices of interest (i.e., honey [36, 37], fruits [38–42], or vegetables [41–47]), great effort has been devoted to both extraction and cleanup procedures that precede instrumental analysis [48]. To obtain satisfactory recovery factors for both neonicotinoids and their main metabolites, several versions of the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method originally proposed by Anastassiades et al. [49] were developed [33, 35, 37, 40, 46].

Although sample pretreatment, preconcentration, and HPLC-MS analysis guarantee good analytical performances—in terms of accuracy, selectivity and instrumental sensitivity—lower limits of detection (LODs; below 1 $ng\,g^{-1}$ or 0.1 ng per bee) were always obtained by applying the optimized analytical method to a large sample: typically 2–15 g, about 20–150 bees. In this way, information on single bee contamination was lost and only an average assessment of the low levels of insecticide uptake was made.

On the other hand, as already mentioned, recent studies have demonstrated that foraging bees can be directly exposed to high (and lethal) concentrations of insecticides in the field. Corn sowing using pneumatic drilling machines and seeds coated with neonicotinoids (an agricultural practice used worldwide) release into the atmosphere large amounts of coating particles that are efficiently intercepted by foraging bees flying over the sowing fields [11, 12, 21, 25, 26]. Bees exposed to these toxic particles show characteristically acute effects, with a short-term mortality which compares well with the colony loss phenomena observed by beekeepers in spring, and associated with corn sowing. Moreover, young corn plants obtained from coated seeds produce guttation drops containing high concentrations of the coating insecticide (up to 1,150 $mg\,L^{-1}$ for thiamethoxam [6, 24]), which are lethal for bees or other insects that may use guttation drops as a source of water. It is worth noting that these acute exposures to neonicotinoids, and their lethal effects on honeybees, can be easily studied and quantified by using dedicated analytical methods based on simpler instrumentation and more rapid procedures than those optimized for the studies of sublethal effects. For instance, neonicotinoids in guttation drops are directly analyzed by ultra-high-performance liquid chromatography (UHPLC) with diode-array detection (DAD) [6, 24], using a rapid method which can also be easily applied (after a suitable sampling procedure) to characterize particulate matter emitted by drilling machines during the sowing of corn coated seeds [11]. The first attempts to use this approach in the analysis of single bees were successful, even if some chromatographic interferences emerged [11]. A UHPLC-DAD method was successfully used in the assessment of acute exposure to seed coating particulate, and consequent lethal contamination, of bees flying close to the drilling machine in the sowing field [21, 25, 26].

In the work reported here, a simplified analytical method was optimized on the basis of QuEChERS extraction and cleanup and UHPLC-DAD instrumental analysis for the accurate determination of neonicotinoid insecticides in single bees. Method validation was also done and analytical performances were assessed by comparing the results with those obtained by an independent UHPLC–quadrupole–time-of flight (Q-TOF)–MS analytical procedure.

## Experimental

### Materials and instrumentation

Analytical grade magnesium sulfate (anhydrous, 99 %; VWR—AnalaR NORMAPUR, Milan, Italy), sodium acetate trihydrate (99.0 %, Fluka, Milan, Italy), Amberlite XAD-2 resin (Restek Ultraclean, Bellefonte, PA, USA), and primary–secondary amine sorbent (PSA, Supelco Supelclean, Milan, Italy) were used in the sample pretreatment step. Methanol (VWR) and acetonitrile (Riedel de Haën, Seelze, Germany) were of HPLC grade, and water was purified using Millipore Milli-Q (Vimodrone, Milan, Italy) equipment. Pure chemicals for instrumental

calibration (Pestanal, purity greater than 99.7 % for thiamethoxam, *N*-desmethyl thiamethoxam, clothianidin, imidacloprid, acetamiprid, and thiacloprid and greater than 97.5 % for fipronil) were purchased from Fluka.

UHPLC-DAD analysis was optimized with a Shimadzu (Milan, Italy) Prominence UFLC-XR chromatograph (SIL 20AC-XR autosampler, CTO-20A column oven, SPD-M20A UV–vis diode-array detector). Liquid chromatography–Q-TOF–MS analyses were performed with a UHPLC system (series 1200, Agilent Technologies, Palo Alto, CA, USA), consisting of a vacuum degasser, an autosampler, and a binary pump coupled with both a diode-array detector and a Q-TOF–MS mass analyzer (Agilent series 6520), equipped with an electrospray ionization (ESI) interface, operating in dual ESI mode, with the following operation parameters: capillary voltage 4,000 V, nebulizer pressure 40 psi, drying gas 10 $L min^{-1}$, gas temperature 350 °C, fragmentor voltage 120 V (180 V in negative ESI mode). On both chromatographic systems, a Shimadzu XR-ODS II analytical column (2.2 μm, 2.0 mm × 100 mm) and a SecurityGuard™ ULTRA cartridge, UHPLC $C_{18}$ 2.1 mm (Phenomenex, Castel Maggiore, Bologna, Italy) guard column were utilized.

## Bee exposure tests

Bees (*Apis mellifera* L.) from four hives, supplied by the Padua Beekeeping Association (A.P.A. Pad), were used in field exposure tests with particulate matter emitted by a drilling machine during corn sowing. All tests were conducted in a sowing field of the experimental farm of the University of Padua (Legnaro, Padua, Italy; coordinates 45° 20′41.19″N to 11°57′16.22″E) using Ribouleau Monosem NG Plus drilling machines under the experimental conditions described elsewhere [11, 25, 26]. Commercially available corn seeds (hybrid X1180D 964890 and PR44G; Pioneer Hi-bred, Italy) produced and marketed in 2010–2011 were used; the seed coatings were Cruiser® (thiamethoxam 0.6 mg per seed, Syngenta, Basel, Switzerland), Poncho® (clothianidin 1.25 mg per seed, Bayer Cropscience, Leverkusen, Germany) and Gaucho® (imidacloprid, 0.5 mg per seed, Bayer Cropscience). A neonicotinoid insecticide in granular form for soil treatment was also used (Santana, containing 0.7 % clothianidin, Sumitomo Chemical Agro Europe, Saint Didier au Mont d'Or, France).

Bees flying over the sowing field, or found dead in the field or close to beehives during the sowing tests, were collected in 1.5 mL test tubes and stored at −80 °C.

## Single bee extraction and cleanup

Sample pretreatment was done by a simplified QuEChERS procedure. In a 1.5 mL test tube, each bee was treated with 100 μL of water and 500 μL of acetonitrile, roughly pounded with a metal pestle, and then 30 mg of magnesium sulfate and 5 mg of sodium acetate were added. The sample was then placed in an ultrasonic bath (ELMA® Transsonic Digitals) for 15 min at room temperature and then centrifuged for 15 min at 10,000 rpm (Hettich MIKRO 120). The supernatant was collected with a syringe, transferred to another test tube, and then 20 mg of PSA sorbent or Amberlite XAD-2 resin was added. To obtain a quantitative recovery of the analytes, the extraction/cleanup process was repeated, treating the bee residue with another 500 μL of acetonitrile; after centrifugation, extracts were pooled, evaporated to dryness at 40 °C under a nitrogen flow, and the residue was dissolved with 300 μL of a water/methanol solution (90:10). The final extract was then centrifuged for 15 min at 10,000 rpm, filtered through a 0.2 μm syringe filter (Phenomenex, RC), and transferred to a 1.1 mL analytical vial.

## UHPLC-DAD analytical method

Compared with the previously optimized procedures [11, 24], a more selective chromatographic method has been developed for the determination of seed coating insecticides in single bees. The UHPLC-DAD instrumental conditions were as follows: eluent flow rate 0.4 $mL min^{-1}$, binary water/acetonitrile gradient elution (eluent A was 90:10 water/acetonitrile, eluent B was acetonitrile; 0–3.5 min, 100 % eluent A; 3.5–14 min, linear gradient from 0 to 12.7 % eluent B; 14–14.5 min, linear gradient to 66.7 % eluent B; 14.5–17.5 min, 66.7 % eluent B; 17.5–18 min, linear gradient to 100 % eluent B; 18–20 min, 100 % eluent B), injector volume 5 μL, and column temperature 45 °C. Detector signals at 278 nm for fipronil, 252 nm for thiamethoxam, and 269 nm for clothianidin and imidacloprid were adopted for quantifying analytes. Thiacloprid and acetamiprid, neonicotinoids that are not used for corn seed coating in Europe, can also be quantified ($\lambda$=244 nm) by the present procedure, along with *N*-desmethyl thiamethoxam ($\lambda$=272 nm), a well known thiamethoxam degradation product [50]. The external instrumental calibration was performed daily by analysis of 50–1,000 $\mu g L^{-1}$ standard solutions of each insecticide in 50 % water/methanol.

## UHPLC-Q-TOF-MS analytical method

UHPLC-Q-TOF-MS analysis used the same elution conditions as previously optimized for the UHPLC-DAD method. The Q-TOF mass spectrometer operated in positive ESI mode for the detection of thiamethoxam, clothianidin, imidacloprid, *N*-desmethyl thiamethoxam, acetamiprid, and thiacloprid and in negative ESI mode for fipronil (ionization mode switching at 17.5 min). Centroid full-scan mass

spectra were recorded over the range 50–1,000 *m*/*z* with a scan rate of two spectra per second. Q-TOF calibration was performed daily with the manufacturer's solution. For all chromatographic runs, *m*/*z* 391.28429 relative to the diisooctyl phthalate molecular ion, which was always present as an impurity residue, was set as the lock mass for accurate mass analysis. The instrument provided a typical resolving power (full width at half maximum) of 18,000 (*m*/*z* 922.0098). Acquisition of mass spectra and data analysis was done with Masshunter B.04.00 (Agilent). External instrumental calibration was performed by analysis of 2–500 ng per bee matrix-matched standard solutions of each insecticide (blank samples fortified after the final filtration step of the optimized extraction procedure). Quantification was done on the basis of the peak area from extracted ion current profiles of the respective $[M+H]^+$ and $[M-H]^-$ (fipronil) ions with a mass window of 0.05 Da.

## Results and discussion

Although rapid methods for the analysis of neonicotinoid insecticides in environmental matrices of interest in the study of colony loss phenomena (i.e., guttation drops and particulate matter) have been optimized recently in our laboratory [11, 24], the direct UHPLC-DAD analysis of methanol or acetonitrile extracts obtained from exposed bees showed some drawbacks, mainly in terms of chromatographic interferences and precipitation of residues (probably waxes) [11, 33, 34,]. On the other hand, specific procedures combining solvent extraction, cleanup and liquid chromatography–MS analysis are quite elaborate and time-consuming, but they undoubtedly guarantee high levels of accuracy and sensitivity that make the analysis of bees exposed to very low levels of insecticides possible. The analytical method proposed in the present work, coupling the advantages of a simplified sample preparation method (QuEChERS) with the possibility of use simpler instrumentation (UHPLC-DAD), can be easily applied to the analysis of neonicotinoid insecticides in single bees after acute exposure, as is the case with the direct contamination of flying bees with corn seed coating particles.

### Optimization of extraction and cleanup procedure

To obtain satisfactory chromatographic selectivity, even at low concentrations, different extraction solvents were tested, i.e., acetone, ethyl ether, dichloromethane, methanol, acetonitrile, and water—in acidic solution (pH 2, by phosphoric acid) too. The first attempts confirmed that acetone and ethyl ether give unsatisfactory recovery factors for neonicotinoids (38–78 % and 10–20 % for acetone and ethyl ether, respectively), whereas dichloromethane showed good recovery factors (74–99 %) but severe matrix interferences mainly affecting clothianidin and imidacloprid determination in most samples. In contrast, water and acidic solutions showed matrix interferences affecting the determination of thiamethoxam (the most water soluble of the neonicotinoids in question). Some of those interfering peaks could be partially removed by liquid–liquid partitioning with *n*-hexane or dichloromethane, which in turn significantly lowers the recovery of the analytes (40–94 % for *n*-hexane, 15–25 % for dichloromethane). The best extraction solvents in terms of both recovery factors and cleanliness from chromatographic interferences were methanol (as previously used in our laboratory) and acetonitrile, which has the advantage to be usable in the QuEChERS extraction technique.

Consequently, a different sample pretreatment approach was studied, starting from well-established QuEChERS methods [33, 35, 37, 40, 46, 49, 51], with some improvements and optimizations in order to apply it, for the first time, to the analysis of single insects. As for the extraction step, different combinations of $MgSO_4$/NaCl and $MgSO_4$/NaOAc aqueous solutions were tested as proposed by Kamel [33]: in our case the results showed negligible differences in terms of recovery factors but an improvement in terms of interfering peaks using $MgSO_4$/NaOAc solutions (see "Experimental" for details).

In any event, the resulting acetonitrile extract could not be directly analyzed by UHPLC mainly because of the presence of substances which are prone to precipitate in column or (clearly) just after dilution with water before instrumental injection. In this connection, dispersive solid-phase extraction (SPE) cleanup using a sorbent such as PSA or Amberlite XAD-2 provided an easy solution, which was quicker than conventional SPE, and ensured very good results: clear extracts, absence of precipitation, and chromatograms that were both highly reproducible and clean from interferences were obtained using both solid phases tested.

Finally, after the evaporation of the solvent, negligible differences were found using different solutions to dissolve the analytes of interest (i.e., water, acidic water solutions, water/methanol, or acetonitrile mixtures); thus a water/methanol solution (90:10) was chosen in order to avoid the unwelcome peak broadening often observed in UHPLC when excess of acetonitrile is injected.

### Optimization of the chromatographic conditions

Taking into account our previously developed procedures [11, 24], we optimized the UHPLC-DAD instrumental conditions in order to improve the chromatographic separation of the selected insecticides from possible matrix interferences, simultaneously shortening the analysis time. The best results were obtained using a water/acetonitrile gradient

elution program, whereas modifiers such as formic acid (0.01–0.1 %), ammonium acetate (0.05 %), and ammonium formate (0.05 %) added to eluents produced a few interfering peaks in DAD partially overlapping with thiamethoxam and clothianidin signals in some samples.

With the optimized UHPLC-DAD method, the elution of five neonicotinoid insecticides and *N*-desmethyl thiamethoxam (a thiamethoxam metabolite) and fipronil (a phenylpyrazole insecticide also used in corn seed coating) takes about 20 min. Of course, if only seed coating neonicotinoids (thiamethoxam, clothianidin, and imidacloprid) are of interest, an anticipated column cleaning step (e.g., at 8.5 min from 6 % to 100 % eluent B in 0.5 min) reduces the analysis time to 12 min.

## Method validation

The performances of the UHPLC-DAD method (summarized in Table 1) were assessed through estimation of accuracy (trueness and precision), sensitivity, selectivity, and linear response range. In view of the impossibility to select real samples (single bees) containing identical concentrations of insecticides, both precision and recovery factors were estimated by analysis of a homogenized pool of non-exposed, lyophilized, and gently powdered bees spiked with known amounts of analytes: 0.03 g portions of this homogenized bee sample (corresponding to the weight of a single lyophilized bee) were added to 50–200 ng of all the analytes (at least four concentrations, two to five samples for each level) and analyzed by the optimized method. The results (i.e., the slopes of the recovery functions, see Fig. S1) evidenced excellent recovery factors: 94±2 % for thiamethoxam, 97±2 % for clothianidin, 87±4 % for imidacloprid, 83±2 % for thiacloprid, 93±1 % for acetamiprid, and 97±2 % for *N*-desmethyl thiamethoxam. Conversely, unsatisfactory recovery was obtained for fipronil (30±3 %), which is a more lipophilic compound than the neonicotinoids analyzed. Precision levels (repeatability) associated with the aforementioned spiked samples were about 5 % for thiamethoxam, *N*-desmethyl thiamethoxam, and thiacloprid, 7 % for clothianidin and imidacloprid, and 10 % for acetamiprid. As expected, the low recovery obtained for fipronil is accompanied by a higher uncertainty (about 50 %). Therefore, the method guarantees both satisfactory recovery factors and good precision levels for each neonicotinoid insecticide, but it shows unsuitable performances for fipronil.

**Table 1** Limits of detection (LOD), repeatability (relative standard deviation), and recovery factors of the entire ultra-high-performance liquid chromatography–diode-array detection analytical procedure for the analysis of neonicotinoid insecticides in single bees

| | LOD (ng/bee) | Repeatability[a] (%) | Recovery factor[a] (%) |
|---|---|---|---|
| Thiamethoxam | 5 | 5 | 94±2 |
| Clothianidin | 7 | 7 | 97±2 |
| Imidacloprid | 7 | 7 | 87±4 |
| *N*-Desmethyl thiamethoxam | 5 | 5 | 97±2 |
| Acetamiprid | 11 | 10 | 93±1 |
| Thiacloprid | 5 | 5 | 83±2 |

[a] From the analysis of bee samples (powdered) spiked with 50–200 ng of each insecticide per bee

The linear response range was experimentally tested for each analyte by instrumental calibration functions up to 100 $mgL^{-1}$ ($r^2>0.999$, $p<10^{-8}$). Method sensitivity (the slope of the calibration function) appears to be similar for each neonicotinoid insecticide, if it is related to mass concentration. Again, a lower sensitivity, which will contribute to a higher instrumental LOD, was observed for fipronil.

The UHPLC-DAD method is characterized by an instrumental LOD of about 8 $\mu gL^{-1}$ for thiamethoxam,

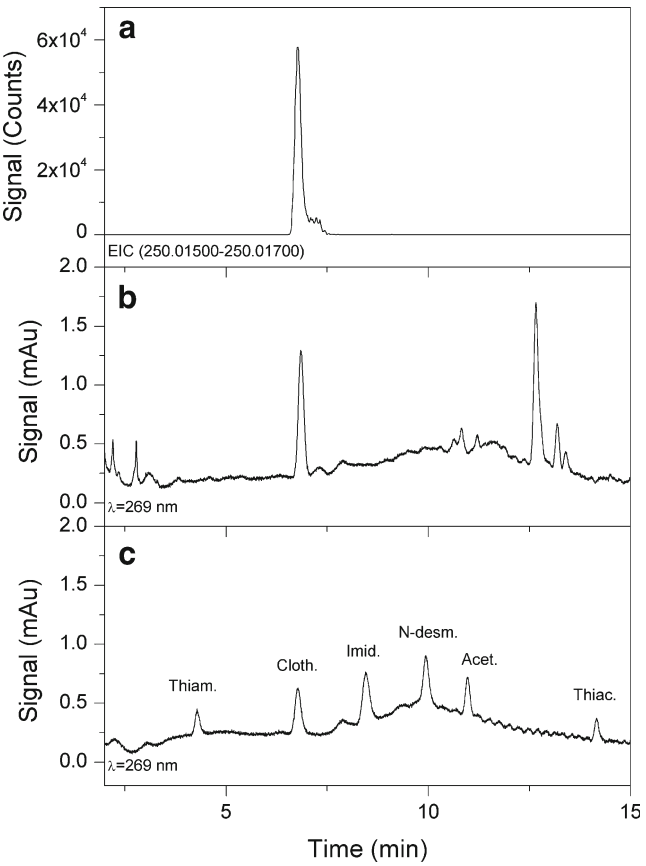


**Fig. 1** Ultra-high-performance liquid chromatography (UHPLC)–quadrupole–time of flight mass spectrometry (**a**) and UHPLC–diode-array detection (**b**) chromatograms of a real sample, a single bee (exposed in the field to seed coating particulate) containing 165 ng of clothianidin. Chromatogram of a standard solution (200 $\mu gL^{-1}$) of each neonicotinoid insecticide (**c**). *Thiam.* thiamethoxam, *Cloth.* clothianidin, *Imid.* imidacloprid, *N-desm.* *N*-desmethyl thiamethoxam, *Acet.* acetamiprid, *Thiac.* thiacloprid

*N*-desmethyl thiamethoxam, clothianidin, imidacloprid, and acetamiprid (13 $\mu g L^{-1}$ for thiacloprid and fipronil), all computed from the parameters of calibration functions using the procedure suggested by IUPAC [52]. In the analysis of real samples by the complete procedure, experimental uncertainties account for actual LODs of 5 ng per bee for thiamethoxam, *N*-desmethyl thiamethoxam, and thiacloprid, 7 ng per bee for clothianidin and imidacloprid, and 11 ng per bee for acetamiprid. As expected, these LODs are significantly higher than those reported for HPLC-MS methods [32–35]. Nevertheless, the UHPLC-DAD method requires simpler instrumentation, easily fitting in common analytical laboratories, and its LODs are adequate for the analysis of single bees exposed to acute levels of neonicotinoid insecticides.

The combination of optimized QuEChERS extraction, dispersive SPE, and UHPLC elution steps efficiently reduces the presence of interfering peaks in the UHPLC-DAD chromatograms of real samples. The absence of chromatographic interferences in the UHPLC-DAD method was further verified by UHPLC-Q-TOF-MS analysis of both spiked and real samples, using identical chromatographic conditions. Monoprotonated and deprotonated molecular ions, attributable to the single analytes, were always obtained as the main peaks for each insecticide in both standard solutions, spiked samples, and real samples. Negligible amounts of sodiated molecular ions were evidenced in both spiked and real samples, and there were no traces of potassiated and ammoniated adduct ions. Analysis of the extracted ion profiles revealed the presence of a small shoulder at a retention time greater than that of the main peak for each insecticide. More selective extracted ion chromatograms performed even at 0.002 Da suggested that these shoulders could be attributable to isomeric forms of these analytes (except for acetamiprid, whose shoulder includes traces of some interfering species). Anyway, the effect of these isomers on the UHPLC-DAD peaks appears to be very limited (Fig. 1).

UHPLC-Q-TOF-MS analysis of real samples made a comparison of the results between two independent instrumental procedures possible. The results from spiked samples ($n$=6, for each neonicotinoid quantified by both detection techniques) were compared by a paired $t$ test, and nonsignificant differences between mean concentrations measured by the two procedures were obtained ($\alpha$=0.05/2, $p$ values of 0.66, 0.25, 0.98, 0.20, 0.053, and 0.09 for thiamethoxam, clothianidin, imidacloprid, *N*-desmethyl thiamethoxam, acetamiprid and thiacloprid, respectively). Also the analyses of single bees (from field exposure tests, $n$=10, results in Table 2) evidenced no significant difference in the experimentally measured concentrations ($p$ values of the paired $t$ test were always higher than 0.25). Our results indicate the possible absence of interferences for the optimized UHPLC-DAD

**Table 2** Concentrations of neonicotinoid insecticides in bees (ng/bee) obtained by the optimized procedure using two independent detection systems: diode-array detection (DAD) and quadrupole–time-of flight mass spectrometry (Q-TOF-MS)

| | Thiamethoxam | | Clothianidin | | Imidacloprid | | *N*-Desmethyl thiamethoxam | | Acetamiprid | | Thiacloprid | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | DAD | Q-TOF-MS | DAD | Q-TOF-MS | DAD | Q-TOF-MS | DAD | Q-TOF-MS | DAD | Q-TOF-MS | DAD | Q-TOF-MS |
| Bee powder spiked with about 50 ng of each neonicotinoid per bee[a] | 49.5±0.3 | 54±9 | 54±3 | 47±4 | 45±1 | 43±1 | 53.2±0.1 | 48±2 | 62±3 | 60±2 | 47.9±0.4 | 57±2 |
| Bee powder spiked with about 100 ng of each neonicotinoid per bee[a] | 83±7 | 87±7 | 97±8 | 96±9 | 74±7 | 70±4 | 94±7 | 91±6 | 98±4 | 93±4 | 81±6 | 79±6 |
| Single bee, exposed in the field to thiamethoxam[b] | 74.9 | 79.3 | | | | | | | | | | |
| | 13.4 | 13.9 | | | | | | | | | | |
| | 6.3 | 5.6 | | | | | | | | | | |
| Single bee, exposed in the field to clothianidin[b] | | | 1,580 | 1,560 | | | | | | | | |
| | | | 2,250 | 2,200 | | | | | | | | |
| | | | 1,220 | 1,160 | | | | | | | | |
| | | | 580 | 610 | | | | | | | | |
| | | | 165 | 158 | | | | | | | | |
| Single bee, exposed in the field to imidacloprid[b] | | | | | 9.4 | 9.1 | | | | | | |
| | | | | | 13.6 | 13.5 | | | | | | |

[a] Average value and standard deviation of two independent measurements

[b] In the analysis of single bees, the estimated uncertainties are 5 % for thiamethoxam and 7 % for both clothanidin and imidacloprid (see the text)

method with satisfactory accuracy in the analysis of single bees exposed to neonicotinoid insecticides.

### Analysis of real samples

The method is currently applied to the analysis of honeybees in both field and laboratory studies aimed at better clarifying the main exposure routes and the real toxicity of these insecticides. Some preliminary results are provided here. First, bees collected in the field after direct exposure to seed coating particulate during corn sowing always show high levels of insecticides, which confirms both our previous observation [11, 25, 26] and the relevance of this exposure–uptake mechanism in the severe colony loss phenomena observed by beekeepers in spring. For instance, in recent sowing experiments using corn seeds coated with clothianidin (Poncho, 1.25 mg per seed, see [26] for details), short-time exposure of caged bees to particulate matter emitted by the drilling machine (about 30 s, simulating one or two flights across the sowing field) gave rise to an effective contamination of 165–2,250 ng of insecticide per bee; these lethal concentrations agree with the levels measured in foraging bees found dead at the beehive immediately after the sowing [11, 21].

Another current study in which our analytical method has been successfully applied deals with degradation mechanisms of neonicotinoids after uptake by bees. In this respect, it is worth noting that spring mortality was often hard to associate with neonicotinoid contamination, mainly because bees found dead in the field or close to the hive exhibited very low concentrations of these insecticides (see, e.g., the bee deaths that occurred in Italy in spring 2008 [53]). As is commonly the case, the sampling–analysis procedure was done some days after the bees had died. Our hypothesis was that a metabolic degradation of the insecticide could significantly affect its real concentration. The first laboratory tests (250–500 ng of thiamethoxam, in alcoholic solutions or adsorbed in talc particles, was deposited on the bee tegument) showed a real degradation, which was more rapid when the bees were alive but was also significant after they had died. Thanks to the present analytical method, we were able to conduct experiments at lower doses (60–125 ng of thiamethoxam per bee, which approaches $LD_{50}$ by contact [9, 54]). We found that bees died within 24 h after we had administered 125 and 60 ng of thiamethoxam per bee, and contained 22–67 and 29–38 ng per bee, respectively (with lower concentrations if the analysis is delayed); but the bees that survived seem to contain a time-dependent decreasing concentration of thiamethoxam, which approached our LOD (5 ng per bee) in about 24 h. Therefore, the degradation of the insecticide that is well documented for sublethal doses in Suchail et al. [55] is present also in bees exposed to lethal doses.

Currently, corn seeds coated with neonicotinoid insecticides are banned in Italy, but these compounds are permitted in spray treatments and, in 2012, also in granular form for soil treatments (i.e., Santana, containing clothianidin). A sowing experiment (spring 2012) using noncoated seeds and Santana under normal sowing conditions indicated that a negligible amount of particles containing the insecticides are released into atmosphere. Indeed, during sowing, the concentration of clothianidin in total suspended particulates sampled at the field margin (and in bees collected in the field too) was always below the LOD of the UHPLC-DAD method.

## Conclusions

The analytical method optimized and validated in this work, based on QuEChERS extraction and cleanup and on UHPLC-DAD instrumental analysis, made the accurate determination of neonicotinoid insecticides in single bees possible and can be easily applied in studies regarding the bee loss phenomena consequent to acute exposure of honeybees to these insecticides in an open field. Its main advantage is its capability to evaluate the uptake variability of individual exposed bees, an important parameter in the assessment of both real exposure and its consequent toxic effects [13]. The method is currently applied in the quantification of new mechanisms of exposure of honeybees to neonicotinoid insecticides and in the study of their degradation processes, both in vivo and post mortem. In this connection, new evidence on the rapid metabolic pathway which occurs in bees after acute exposure to these insecticides could explain the remarkable lack of insecticides often detected in bees collected in the field some days after their death.

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