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Residues of neonicotinoids and their metabolites in honey and pollen from sunflower and maize seed dressing crops

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ABSTRACT

A study was carried out to evaluate the possible presence of thiamethoxam, clothianidin and imidacloprid, as well as the metabolic breakdown products of these three neonicotinoids in pollen and honey obtained from brood chamber combs of honeybee colonies located next to sunflower and maize crops from coated seeds. Samples were analyzed by liquid chromatography coupled to quadrupole-time-of-flight mass spectrometry detector, in combination with accurate mass tools such as diagnostic ions by exact mass, chlorine mass filters, and MS/MS experiments. The presence of thiamethoxam and clothianidin was confirmed in some of the pollen samples analyzed. Moreover, different metabolites of neonicotinoids were tentatively detected in the pollen and honey samples collected. The results suggested that four metabolites were found in the honey samples, while for pollen samples eleven metabolites were identified; among these, five were considered for the first time as metabolic breakdown products in sunflower and maize plants.

1. Introduction

Due to their worldwide application, neonicotinoids are producing a serious public concern [1] which has been reflected in a large number of articles devoted to their study by research groups in many countries, as may be confirmed by the recent conclusions of the Worldwide Integrated Assessment group [2].

A large part of neonicotinoid use consists of seed coating application into the soil where they are to be taken up by plants, e.g., by roots and then transported along the phloem or the xylem to distal tissues different from those where the product was applied [3], including the flowers [4], their pollen [4–6] and nectar [6,7]. Thus, it does no matter where a pest or non-target organisms (such as those feeding on plant material, and those collecting nectar and pollen) attack the treated plant because it is likely to come in contact with these chemicals [1,8]. As an example, Krupke et al. [5] reported that dead bees found near hive entrances contained the neonicotinoid clothianidin. These findings have been related with honeybee's intoxication when are exposed to these insecticides used as seed treatments and with their difficulty to find the way back to the hive, leading to their depopulation and what is known colony collapse disorder (CCD) [9,10], a conclusion which, nevertheless, is not supported by all researchers [11].

Neonicotinoids undergo an intense metabolism in plants, leading to the appearance of different metabolites during the plant's life or up to the harvesting time of a plant consumed by humans or breeding animals [12]. Metabolites of neonicotinoids have been found in various media, plants and organisms, and studying their presence is currently a topic of interest, because they have been described as molecules with higher toxicity than the neonicotinoids, resulting in a more prolonged harmful effect [13,14]. Thus, the presence of a neonicotinoid together with some of its metabolites in environmental matrices may even increase their toxic action.

These phenomena have recently led to the European Union's taking action by adopting a regulation to restrict the use and sale of seeds treated with plant protection products containing three particular neonicotinoids: clothianidin, imidacloprid and thiamethoxam [15]. Hence, we have focused our work in those three compounds as well as on the possible metabolites, and trying to ascertain their presence in beehives located near fields where maize and sunflower coated seed was used before the restriction, whilst analyzing the pollen and honey collected in brood chamber combs. To the best of our knowledge, some metabolites

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of clothianidin and imidacloprid have been studied in pollen [16–18] and certain imidacloprid metabolites have been examined in honey [19]; however, only four metabolites were considered for clothianidin and up to six were simultaneously considered for imidacloprid. No data exists regarding the search for metabolites of thiamethoxam in these matrices.

Advanced high-resolution mass spectrometric (HRMS) platforms have been considered as suitable instruments for multiresidue pesticide analysis and identification, however, its coupling with liquid chromatography by electrospray interface (LC–ESI–MS/MS) has become the most widely used technique for determining pesticides in environmental and food samples [20,21]. The HRMS analyzers used have been: time-of-flight (TOF), quadrupole-time-of flight (QTOF), Orbitrap and tripe quadrupole mass spectrometers. Among them, QTOF offers high accuracy for the identification of unpredicted, unknown species and benefits from the increased resolving power of m/z signals in comparison to other analyzers such as single and triple quadrupole [22], facilitating the measurement of accurate masses of ions and showing characteristic isotopic patterns of chlorine-containing species.

Since information regarding the residues of thiamethoxam, clothianidin, imidacloprid and their metabolites in honey and pollen is not available in Spain as a result of European Union action, in this study the LC/QTOF–MS technique in combination with accurate mass tools has been used for analyzing honey and pollen samples collected from apiaries close to crops of maize and sunflower grown from seeds coated with these neonicotinoids. The aim of the study is to detect the presence of these in the given samples and to characterize their possible metabolites.

2. Experimental

2.1. Reagents and chemicals

Neonicotinoid insecticide standards (clothianidin, imidacloprid and thiamethoxam) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Methanol, acetonitrile, and acetone were supplied by Lab Scan Ltd. (Dublin, Ireland). Formic acid (98–100% pure) was obtained from Sigma–Aldrich Chemie Gbmh (Steinheim, Germany). A Vibromatic mechanical shaker and ultrasonic bath (Ultrasons) supplied by J.P. Selecta S.A. (Barcelona, Spain), a 5810 R refrigerated benchtop centrifuge from Eppendorf (Hamburg, Germany), a Moulinette chopper device from Moulinex (Paris, France), and an R-210/215 rotary evaporator from Buchi (Flawil, Switzerland) were used for all extractions. Nylon syringe filters (17 mm, 0.45 μm) were acquired from Nalgene (Rochester, NY,USA), and ultrapure water was obtained using Milipore Mili-RO plus and Mili-Q systems (Bedford, MA, USA).

Standard stock solutions were prepared by dissolving approximately 1 mg of each neonicotinoid insecticide, accurately weighed, in 10 mL of acetone. These solutions were further diluted with a water and acetonitrile (50:50, v/v) mixture in order to prepare the working solutions. All standard solutions were kept in the dark at −20 °C.

2.2. Instrumentation and conditions

An UPLC system (ACQUITY UPLC, Waters, Milford, MA) and a Quadrupole-time-of-flight (QTOF) mass spectrometer (maXis impact, Bruker Daltonik GmbH, Bremen, Germany) were coupled through an electrospray interface.

Analyses of samples were conducted by means of a slightly modified HPLC–MS method developed in the research group [23]. Briefly, chromatographic separation was performed on a fused-core type column (Kinetex® 2.6 μm C18, 150 mm × 4.6 mm i.d.), protected by a C18 guard column (4 mm × 2.0 mm i.d.); both were obtained from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) applied at a flow rate of 0.5 mL/min in the following gradient mode: (i) 0 min (A–B, 90:10, v/v); (ii) 8 min (A–B, 65:35, v/v); (iii) 13 min (A–B, 60:40, v/v); (iv) 14 min (A–B, 0:100, v/v); (v) 15 min (A–B, 0:100, v/v); (vi) 16 min (A–B, 50:50, v/v); (vii) 17 (A–B, 90:10, v/v); and (vii) 24 (A–B, 90:10, v/v). Injection volume and column temperature were set at 50 μL and 25 °C, respectively. The mass spectrometer was operated in the positive ionization mode because it provided the greatest sensitivity for the neonicotinoid insecticides, with a capillary voltage of 3500 V, a flow rate of drying gas (N_2) of 9 L/min at a temperature of 340 °C, and a nebulizer gas (N_2) pressure of 4 bar. Spectra were acquired in a mass range of mass/charge (m/z) 50–600. The m/z scale of the mass spectra was calibrated daily by infusing a 10 mM sodium formiate solution. MS/MS fragmentation was carried out in multiple reaction monitoring (MRM) mode by using an isolation width of 5 m/z and a collision energy ramp from 10 to 30 eV. MRM analysis consisted of the precursor ions were selected simultaneously in order to obtain their full MS/MS spectrum in one run. A window of ±0.02 m/z for the extracted ion chromatograms (EIC) was used in order to extract the exact mass. Recorded data was processed with software Data Analysis 4.1 and Qualitative Analysis from Bruker Daltonik GmbH.

2.3. Samples

Samples were provided by the Regional Apiculture Center of Marchamalo (Guadalajara, Spain) in 2013. Honey samples were extracted from brood chamber combs of beehives located close to crops of sunflower from thiamethoxam coated seeds (samples HS1–HS5), and crops of maize from thiamethoxam, clothianidin and imidacloprid coated seeds (samples HM1–HM4) located in the Central Region of Spain. These crops had an experimental grown. The plots were treated with preemergent herbicides (2.5–4 L/ha, Aclonifen 60% p/v Challenge-Bayer) and the seeds were treated in the laboratory with the neonicotinoids (1 L/Qm seed: Focus, RAGT, thiamethoxam; Poncho 600FS, Bayer, clothianidin; Confidor 20%, Bayer, Imidacloprid). The hives were located in the central part of the crops to ensure the presence of worker-bees on them. Prior to treatment the samples from each apiary were pooled and then centrifuged at 12,700 × g for 30 min at 20 °C to remove residual solid particles.

Pollen samples were taken from different beehives in the same apiaries located close to crops of maize from seeds treated with thiamethoxam, clothianidin and imidacloprid (PM1–PM7) and close to crops of sunflower with seeds coated with thiamethoxam (PS1–PS26). The collected pollen samples were homogenized by grinding and dried at 45 °C in an oven.

Honey and pollen samples, obtained of brood chamber combs from beehives located in areas where no insecticide treatment had ever been applied, and collected in the same days as those located near to maize and sunflowers crops, were the object of a preliminary analysis by LC–ESI–MS/MS in order to verify the absence of neonicotinoids and their metabolites. Once this absence was confirmed, they were used as blank samples.

2.4. Sample treatment

In the case of honey, 5 g of sample was dissolved in 10 mL of water, following which a solid phase extraction (SPE) using reverse phase polymeric cartridges (Strata X 33 μm Polymeric Sorbent with 250 mg of sorbent) was optimized. The cartridges were first conditioned with 5 mL of methanol and then equilibrated with 5 mL of water. The sample was then loaded onto the cartridges and the sample flow-through was discarded since early trials showed that