

Detection and quantification of boscalid and its metabolites in honeybees

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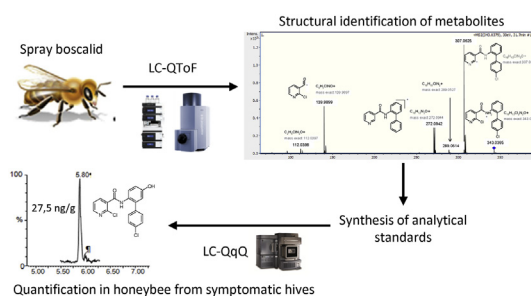
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HIGHLIGHTS

- Methodology based on the use of both high- and low-resolution mass spectrometry.
- Identification for the first time of boscalid metabolites in honeybees.
- Quantification of boscalid and three metabolites in honeybees collected in hives.

GRAPHICAL ABSTRACT



ABSTRACT

Boscalid is a new-generation fungicide that has been detected in several bee matrices. The objective of this work was to characterize boscalid metabolites in honeybees based on *in vivo* experimentation, and next to verify the presence of these metabolites into honeybees from colonies presenting troubles. A methodology based on complementary mass spectrometric tools, namely ultra-high performance liquid chromatography coupled to high-resolution mass spectrometry (UHPLC-QToF) or triple quadrupole mass spectrometry (UHPLC-QqQ) was implemented. Honeybees were sprayed with boscalid, at field rate (to induce the metabolization process) and the parent compound with its generated metabolites were then extracted using modified EU-QuEChERS method. The mass characteristics including exact mass, isotopic profile and mass fragments allowed assuming the structure of several metabolites. Some of them were unambiguously identified by comparison with synthesized analytical standards. The metabolites were resulted from hydroxylation and dechlorination of the parent compound as well as the substitution of a chlorine atom with an hydroxyl group. The metabolites were then quantified in bee samples collected from various beehives located in France. Boscalid and three of its metabolites were present in some samples at a level ranged between 0.2 and 36.3 ng/g.

Keywords:
Boscalid
Honeybee
Metabolite
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Quantification

1. Introduction

Bees have been affected worldwide for twenty years by excess mortality. A combination of factors may explain this phenomenon as the decline in floral diversity, chronic exposures to pesticides or

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stress induced in particular by viruses or parasites (Stokstad, 2007; Ratnieks and Carreck, 2010; Goulson et al., 2015). The role of plant protection products in the weakening of pollinating insects is a proven fact. However it is difficult to demonstrate a relationship between the deleterious effects induced by pesticides and exposure to them.

One difficulty lies in the fact that the search for pesticides in bees involves parent target substances. However these may have been degraded by abiotic processes or metabolized by bees especially in case of delayed mortality findings; thus, the parent compound may no longer be detectable in the bee or at very low levels. The very few toxicokinetic available data make difficult the interpretation of results. However, these data are necessary to conclude both on the presence of the suspected parent molecule and on the initial contents that have induced detrimental effects. Evidence of contact between bee and pesticide may, nevertheless, be obtained by detecting metabolites or markers of exposure in bees. On the other hand, the study of metabolites and their toxicity to the honeybee, which may be greater than that of the parent molecule, is still undeveloped.

To date, there is a limited number of studies on the metabolism of pesticides in bees (Suchail et al., 2004; Brunet et al., 2005), although several types of enzymes have been identified as capable of metabolizing xenobiotics in the bee. Indeed phase I enzymes catalyze functionalization reactions whereas phase II enzymes catalyze conjugation reactions (Suchail et al., 2004; Xu et al., 2013). However, given the levels of exposure to which bees are subject, methods of analysis are neither, in many cases, adapted nor sensitive enough to detect and quantify the metabolites. Other challenges are related to the lack of information on the nature of the metabolites and moreover to the lack of analytical standards, which are essential for conducting the relevant pesticide analysis. It is, therefore, necessary to undertake studies on the degradation of pesticides in bees to enable diagnoses of bee poisoning consecutive to plant protection treatments.

The pesticide selected for this study is the broad spectrum fungicide boscalid (Fig. 1), a new generation compound of the carboxamide family that acts by inhibiting fungal respiration by blocking the ubiquinone-binding sites in the mitochondrial complex II involved in the Krebs cycle (Aveno and Michailides, 2007). Since its placing on the market in 2002, boscalid has been intensively applied, mainly during the flowering period, for crop protection throughout the plant growth, especially for the control of white mold and foliar diseases in vineyards or in fruits and vegetables such as corn, carrots, cabbage, beans or peas. Therefore honeybee is likely to be in contact with this fungicide. Some recent works of David et al. (2015, 2016) have revealed the presence of boscalid in bumblebees (up to 9.8 ng/g) and pollens (up to 38 ng/g). Near $\mu\text{g/g}$ levels of the fungicide were reported in wax samples or pollen (Mullin et al., 2010; Jabot et al., 2015). Ostiguy and Eitzer (2014) quantified the fungicide in an overwintered honey sample (2.3 ng/g).

The main objectives of our study were (1) the identification of the metabolites of boscalid in honeybees, based on an *in vivo* biotransformation following an experimental spraying of the fungicide and (2) the quantification of these metabolites in bees from various hives in France.

To achieve these objectives, we have implemented analytical strategies based on the use of the coupling of ultra-high performance liquid chromatography (UHPLC) with two different mass spectrometers: a time of flight (QqToF) and triple quadrupole (QqQ) analyzers. The QqToF technology was used for its ability to determine the accurate mass and the formulas of by-products. Moreover, the use of MS/MS fragmentation has helped in identifying new or hypothesized metabolites. The QqQ was used for its selectivity and sensitivity to quantify the identified metabolites, in a complex matrix such as bees.

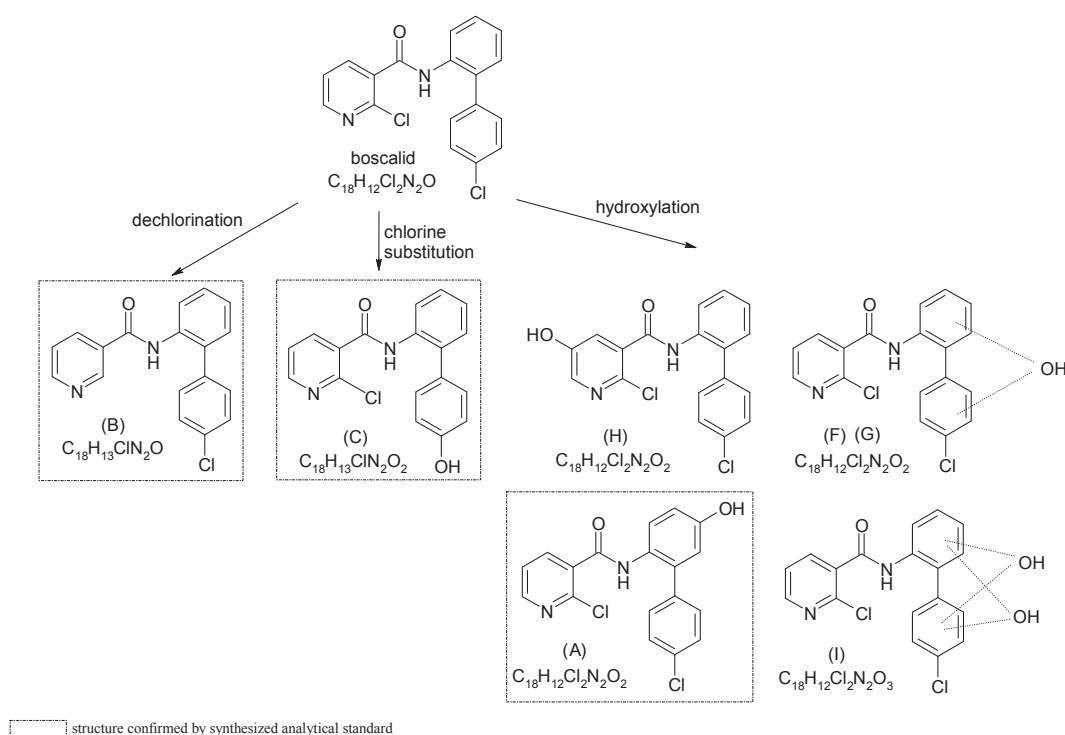


Fig. 1. Structure of boscalid and the identified or predicted metabolites.

2. Materials and methods

2.1. Chemicals

The analytical standard of boscalid [2-chloro-N(4'-chlorobiphenyl-2-yl)-nicotinamide], CAS registry n°188425-85-6 was obtained from Sigma-Aldrich (Saint Quentin Fallavier, France) with a >99% purity. Five analytical standards A (>95% purity), B (>98% purity), C (>99% purity), D (>99% purity) and E (>96% purity) in Figs. 1 and 2 were synthesized by DiverChim SA (Roissy-en-France, France). A storage solution (20 mL) of each of the six standards was prepared at a concentration of 1000 mg/L in acetonitrile (ACN) and was stored at -18°C . The solutions used for the development of the separation and the MS/MS fragmentation experiments were prepared by the dilution of these stock solutions in deionized water.

Cantus[®] commercial product from BASF was used for the spraying test. It contained 500 g/kg of active ingredient and was in the form of dispersible granules. The spray solution was prepared by dilution of the commercial product in water/acetone (99/1; v/v) so as to obtain a boscalid concentration of 5 g/L.

ACN, methanol (MeOH) (UPLC-MS grade) and ammonium acetate were obtained from Biosolve Chimie (Dieuze, France). Heptane (LC grade), formic acid and triethylamine were furnished by Sigma-Aldrich. Deionized water was obtained from a MilliQ device from Millipore (Saint-Quentin-en-Yvelines, France). Primary secondary amine (PSA)/C18 dispersive phase was purchased from Macherey-Nagel (Hoerd, France).

2.2. Boscalid spraying

The honeybees (*Apis mellifera*) were collected from the reserve frames of the hive body from a colony in the experimental apiary of INRA (UR 406 A&E Avignon, France) which health status was regularly controlled. Bees were carefully collected and then anesthetized by inhalation of carbon dioxide for 5 min before being distributed in ventilated cages by groups of 30. The cages were then placed in the dark at $28 \pm 2^{\circ}\text{C}$ for 24 h, with a humidity rate of $60 \pm 10\%$. Bees were fed *ad libitum* with water and a syrup composed of micro-crystals of saccharose (83%) coated with fructose and glucose. Bees were anesthetized again and placed on rotating discs in a Potter-Burgerjon-type tower. The discs were then exposed to the spraying of the Cantus[®] spray solution to obtain a field rate of 1 kg/ha. An amount of 120 bees were taken and frozen immediately after the spraying (T0bosc). After that, 120 other bees were placed again in cages, at $28 \pm 2^{\circ}\text{C}$. After 24 h, the living bees were put at -20°C to stop the metabolization (T24bosc). Control sets were made during this experiment: T0bee (120 bees frozen just after the collect in the hive and drowsiness) and T0wat (120 bees frozen after spraying with distilled water to reproduce the stress and, therefore, the physiological changes suffered during spraying). These experiments were performed in triplicate.

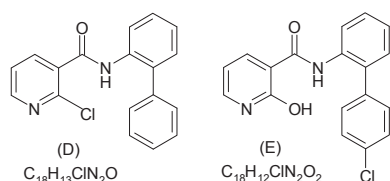


Fig. 2. Structures of the synthesized compounds D and E.

2.3. Analysis of *in vivo* honeybee extracts for metabolites identification

A QuEChERS-based extraction (liquid-liquid extraction with acetonitrile assisted by salts) was performed to extract the compounds from the honeybees (EN-15662, 2008). A mass of 5 g of honeybee was weighed in a 50 mL polypropylene centrifuge tube containing stainless steel beads. Volumes of 3 mL of pure water, 3 mL of heptane (used to remove lipids) and 10 mL of ACN/triethylamine (98/2; vol/vol) were introduced into the tube. The mixture was then vigorously shaken during 15 s. A citrate buffer packet from Agilent Technologies (Massy, France) was added, and the tube was immediately manually shaken again for 10 s and swirled on a rotative mixer for 20 s to homogenize the sample. The mixture was then centrifuged for 2 min at 7000g. A 8 mL aliquot of the supernatant (ACN phase) was transferred to a 15 mL tube and incubated for 15 h at -18°C . Afterwards, a 6 mL volume of the extract was transferred to a 15 mL centrifuge tube containing 150 mg of PSA/C18 dispersive phase from Macherey-Nagel (Hoerd, France). The tube was swirled on a rotative mixer for 10 s then the extract was centrifuged again (7000 rpm, 2 min) and 4 mL of the supernatant were transferred to a glass tube. Then, the solvent was evaporated to dryness under a gentle stream of nitrogen at 40°C . The dry residue was dissolved with 320 μL MeOH and 80 μL of 25 ng/ml phenacetin solution (25 $\mu\text{g}/\text{L}$) used to control the injection accuracy. Lastly, 40 μL of this solution were added to 160 μL of deionized water for the UHPLC-QToF analysis.

Liquid chromatography was performed using a UHPLC Ultimate 3000 system from Thermo Scientific (MA, USA). The separation was carried out with a Kinetex Phenyl-Hexyl (100 \times 2.1 mm, 2.6 μm) column from Phenomenex (Le Pecq, France). The mobile phases were (a) 0.01% acetic acid with 0.4 mmol/L ammonium acetate in deionized water and (b) MeOH and were applied using the following gradient: from 5% to 90% (b) in 45 min. The flow rate was fixed at 0.4 mL/min, the oven temperature at 60°C , and the injection volume was 2 μL .

The chromatographic system was coupled to a MicroTOF-QII mass spectrometer from Bruker Daltonics GmbH (Bremen, Germany). Electrospray ionization was performed in the positive mode with the following parameters: capillary voltage 4500 V, source temperature 200°C , nitrogen desolvation gas flow 900 L/h and 660 L/h and nitrogen nebulization gas pressure 2.8 bars. Full scan data were acquired from 50 to 1000 Da and between 340 and 540 Da in order to improve the sensitivity. The mass spectrometer was calibrated using a solution of sodium formate (10 mL NaOH 0.1 M + 250 mL isopropanol + 10 μL formic acid). MS/MS fragmentation was performed at three collision energies (10, 20 and 30 eV, respectively). The software Data Analysis (Bruker Daltonics GmbH) in its 1.3 version was used for the data treatment.

2.4. Bee samples

Honeybees used as blank matrix for matrix-matched calibration were collected from the Ouessant Island situated in the Atlantic Ocean. Their previous analysis confirmed the absence of boscalid and metabolites. A total of 37 honeybee samples were collected by beekeepers during the 2015 beekeeping season from various hives located in France. The sampling was achieved further to a report of either bee disorders in the hive or bee death. All blank and honeybee samples were stored at -20°C until analysis.

2.5. Quantification of boscalid and metabolites in honeybees

The same QuEChERS-based extraction previously described was performed to extract the target compounds from the honeybees.

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Liquid chromatography was performed using an H-Class UPLC system from Waters (Saint Quentin en Yvelines, France). The separation was carried out with the column, mobile phases, flow rate and oven temperature previously described. The injection volume was 5 μ L. The following gradient was applied: from 5% to 90% (b) in 7 min, followed by 100% (b) for 2 min. The column was then equilibrated at the initial conditions for 2 min.

The chromatographic system was coupled to a Xevo TQ-S triple quadrupole mass spectrometer from Waters. Electrospray ionization was performed in the positive mode with the following optimized parameters: capillary voltage 3200 V, desolvation temperature 450 °C, source temperature 150 °C, nitrogen desolvation, and nebulizer gas flows 900 L/h and 150 L/h, respectively. For each compound, two multiple reaction monitoring (MRM) transitions were optimized, based on their infusion into the mass spectrometer and the use of IntelliStart™ that automatically selects the *m/z* values for the precursor and product ions. The target ion transition with the highest signal intensity (MRM1) was used for quantitation, whereas the second target ion transition (MRM2) was used for confirmation. The ion transitions, cone voltages, collision energies and dwell times of the analytes are displayed in Table 1. The limit of quantification (LOQ) was calculated by spiking the blank matrix with a mixture of boscalid and the five synthesized compounds to get the levels 0.25, 1, 2, 5, 10 and 20 ng/g. The LOQ corresponded to the content for which a signal-to-noise ratio of 10 was obtained for MRM1 with a signal-to-noise ratio of at least 3 for MRM2.

3. Results and discussion

3.1. Sample preparation

The identification of metabolites from *in vivo* experimentation was difficult due to the nature and the complexity of the matrix. The analytical challenge lied in the development of a sample preparation that efficiently enabled extracting the potentially generated metabolites while limiting the presence of interfering substances that may cause signal suppression during the analysis or complexify the chromatograms. The sample preparation for the analysis of boscalid and its metabolites was derived from our previous work on honeybee (Wiest et al., 2011). Compared to this protocol, based on a EU-QuEChERS extraction, 2% of triethylamine were added to acetonitrile as metabolites with acidic functions could be generated. Indeed, Kamel (2010) found that pesticides containing carboxylic acid groups were retained by the PSA-type purification phase. The author recommended the use of triethylamine, a strongly polar base, before the purification step to recover acidic compounds.

Table 1
Retention times (Rt), transitions used for the quantification (MRM1) and confirmation (MRM2), and source parameters of boscalid and five synthesized standards analyzed by LC-QqQ.

Compound	Rt (min)	Transitions	Cone voltage (V)	Collision energy (eV)
Boscalid	6.5	MRM1 343.2 \rightarrow 307.1	30	20
		MRM2 343.2 \rightarrow 140.1	30	20
A	5.8	MRM1 359.2 \rightarrow 139.9	10	20
		MRM2 359.2 \rightarrow 323.1	10	20
B	6.4	MRM1 309.2 \rightarrow 105.9	12	34
		MRM2 309.2 \rightarrow 80.3	12	24
C	5.5	MRM1 325.2 \rightarrow 140.0	12	16
		MRM2 325.2 \rightarrow 112	12	38
D	6.3	MRM1 309.3 \rightarrow 140.0	16	16
		MRM2 309.3 \rightarrow 273.1	16	16
E	6.8	MRM1 325.2 \rightarrow 122.0	24	20
		MRM2 325.2 \rightarrow 94.0	24	38

3.2. Analytical strategy to detect and identify the metabolites

Several approaches were used to detect and identify the potentially generated metabolites of boscalid. The first strategy consisted in establishing a list of boscalid metabolites previously identified in the literature in different media or organisms such as rats (Grosshans and Knoell, 2001) or soil (US EPA, 2003). We also included transformation products from electrochemical, photolytic or photocatalytic reactions (Lohmann et al., 2009; Lagunas-Allué et al., 2010; Lassalle et al., 2014). The exact mass of each potential metabolite was calculated as well as several adducts, namely $[M+H]^+$, $[M+Na]^+$ and $[M+NH_4]^+$. The mass spectrometer was first operated in single MS full scan mode. All the above calculated masses were extracted from the total ion chromatograms (TIC) of the extracts corresponding to the bees exposed to boscalid and to control samples. Thus, a total of 195 masses of known metabolites and adducts were searched in the TICs. If the extraction of these masses brought up a peak on the chromatogram, one or several proposals of empirical formula were generated by Smart Formula (Bruker Daltonics GmbH), each of them being associated with a theoretical exact mass and a theoretical isotope pattern. The comparison between theoretical and experimental data (mass difference Δm expressed in ppm and isotopic pattern difference in *mSigma*) could confirm or exclude the proposed empirical formula. To establish an empirical formula, it is recommended to be within a difference of less than 5 ppm and isotopic pattern fit factor *mSigma*, respectively. If the generated molecular formula was acceptable, the corresponding masses were extracted in the same way, in the TIC of control samples to verify that the molecule was generated during the time of metabolization.

The second strategy was to search for chlorinated molecules as boscalid contains two chlorine atoms. Indeed, from the characteristic abundance of $^{35}\text{Cl}/^{37}\text{Cl}$ (100/31.98), the isotopic pattern of a chlorine-containing molecule is typical. It was thus possible to extract the masses presenting this particular pattern. An empirical formula containing one or two chlorine atoms was assigned. As mentioned above, the comparison between the exact theoretical mass and the exact experimental mass confirm or reject the empirical formula. If the mass was retained, its presence was checked in the TIC of the control samples. To provide additional information on the structure of the metabolite, MS/MS fragmentation was then performed in both approaches. Finally, some metabolites tentatively identified were synthesized and analyzed by MS/MS with the same chromatographic and mass conditions than the extracted honeybees so as to compare the retention times and mass fragments and consequently to confirm or invalidate their identities.

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3.3. Identification of the metabolites

The duration of the metabolization was chosen from kinetic data obtained by Poquet (2015) during the metabolic study of bees exposed to pesticides belonging to various chemical families. The results showed a significant decrease (80%) of the concentration of the parent molecules in the first 24 h, for most of the studied pesticides. Therefore, a 24 h metabolization time was set for boscalid.

All the samples and controls (T0bee, T0wat, T0bosc, and T24bosc) were analyzed in full scan mode by UHPLC-MS. The full scan mass spectrum of the samples T0bosc and T24bosc showed a pseudo-molecular ion $[M+H]^+$ at m/z 343.0385 which corresponded to $\Delta m = 4.1$ ppm compared to the standard of boscalid, with the same retention time ($R_t = 31.7$ min). The MS/MS fragmentation of boscalid (Fig. 3, Table 2) showed several fragment ions. The fragment ion at m/z 307.0625 described the loss of one chlorine atom from the protonated boscalid (calculated exact mass 307.0632) while the m/z 272.0942 corresponded to the loss of two chlorines (calculated exact mass 272.0944). The fragment ion at m/z 289.0514 was due to the loss of H_2O of the dechlorinated protonated boscalid (calculated exact mass 289.0527). The fragment ion at m/z 139.9899 could be attributed to the 2-chloropyridine-4-carboxy moiety of boscalid (calculated exact mass 139.9897). Finally, m/z 112.0398 could be ascribed to $C_2H_9ClN_2O^+$ (calculated exact mass 112.0397). These fragments may represent indications to identify boscalid metabolites.

The masses corresponding to the 195 theoretical metabolites or adducts were extracted from the sample T24bosc. Five of them were present in the TIC, namely m/z 359.0337, 359.0341, 359.0338 and 359.0358 (four peaks with various retention times) and m/z 375.0303 (one peak).

The masses m/z 359.0337, m/z 359.0341, m/z 359.0338 and m/z 359.0358 correspond to the addition of one atom of oxygen into boscalid. This was in line with the retention times of the 4 compounds whose masses were inferior to that of boscalid (Table 2), indicating the formation of more polar compounds than boscalid itself, such as hydroxylated compounds. The chemical structure of boscalid (Fig. 1) is composed of 3 moieties: a pyridine ring, and two phenyl rings. The hydroxylation could, therefore, occur on several sites in these cycles.

The main mass fragment ion of the hydroxylated-boscalid with

$R_t = 29.6$ min was m/z 155.9842. It is correlated to the 2-chloropyridine-4-carboxy fragment of boscalid to which an atom of oxygen ($139.9897 + 15.9943 = 155.9840$) was added. This indicated a hydroxylation on the pyridine ring of boscalid. This hypothesis was supported by the presence of m/z 113.0038 corresponding to the 2-chloropyridine-hydroxy- fragment of boscalid. As CONH group induces a meta-substitution on the ring, whereas chlorine directs substitution in ortho- or para-, it is likely that the hydroxylation took place in position 3 of the pyridine ring (compound H in Fig. 1). The hydroxylation of the pyridine moiety was previously identified as a metabolic pathway of boscalid in rats (Grosshans and Knoell, 2001) but the localization on the cycle was not elucidated.

The mass fragment m/z 323.0572 (or m/z 323.0573) appeared in the MS/MS fragmentation of the 3 other hydroxylated compounds, namely A, F, and G in Table 2 and Fig. 1. This mass can be attributed to $C_{18}H_{12}ClN_2O^+$ ion with an added oxygen atom ($307.0633 + 15.9943 = 323.0576$). The 3 hydroxylated compounds A, F and G showed also the same m/z 139.9897 mass fragment, characteristic of the unmodified pyridine ring, and the absence of m/z 155.9840 and 113.00 fragments. This would, therefore, suggest hydroxylation on one of the benzenic rings. However, the MS/MS fragments (Table 2) did not allow concluding on the exact structure of the molecule. The hydroxylated compound A was synthesized and both its chromatographic and mass spectral characteristics were compared to the signals of the extracts T24bosc. The identification of [2-chloro-N (4-hydroxy-4'-chloro-biphenyl-2-yl)-nicotinamide] with retention time $R_t = 25.4$ min was thus unambiguously confirmed. This hydroxyl-boscalid was previously identified as one of the main metabolization product of boscalid in rats, after oral administration, together with its glucuronic acid conjugate (Grosshans and Knoell, 2001). In the honeybee extract T24bosc, this conjugated compound was not detected.

The mass $m/z = 375.0303$ corresponded to $[M+H]^+$ adduct of boscalid with two hydroxylated groups ($343.0399 + 2 \times 15.9943 = 375.0285$). The presence of m/z 139.9903 together with the absence of signal at m/z 155.9840 during the MS/MS fragmentation may suggest that hydroxyl groups were positioned on the aromatic rings (I in Fig. 1). The other fragment ions did not allow going further in identifying the structure (Table 2).

Besides these hydroxylated derivatives of boscalid, other masses

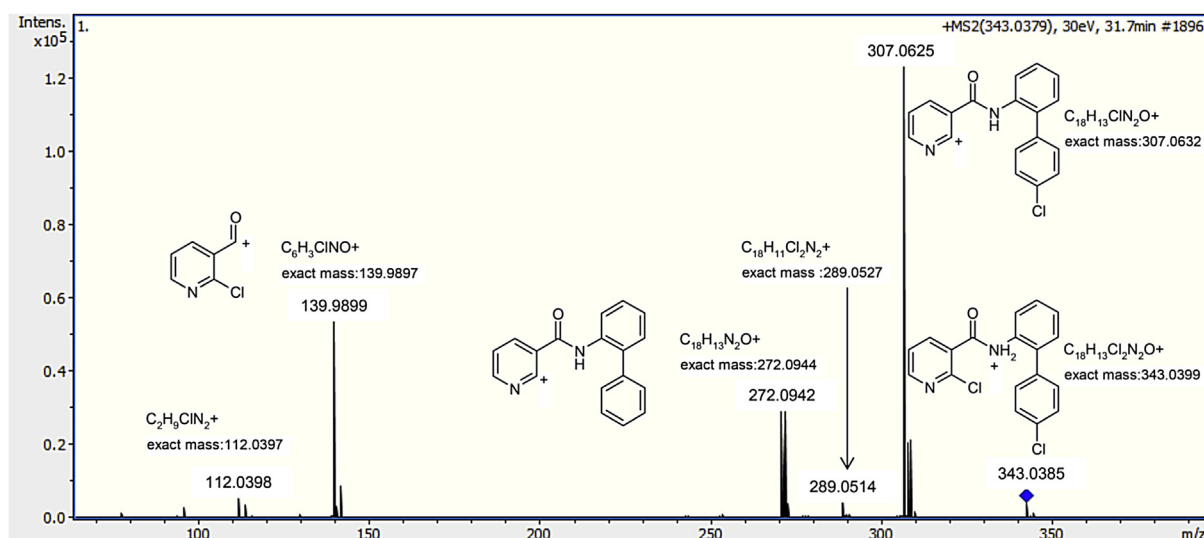


Fig. 3. MS/MS fragmentation spectrum of boscalid in bee extract (collision energy 30 eV).

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Table 2

Retention time (Rt), empirical formula, pseudo molecular ion [M+H]⁺ and MS/MS mass fragments of boscalid and its metabolites in the sample T24bosc.

Pseudo-molecular ions						MS/MS fragment ions			
Compound	Rt (min)	Empirical formula	Exp. mass (Da)	Theo. mass (Da)	Error (ppm)	Empirical formula	Exp. mass (Da)	Theo. mass (Da)	Error (ppm)
Boscalid	31.7	C ₁₈ H ₁₃ Cl ₂ N ₂ O	343.0385	343.0399	-4.1	C ₁₈ H ₁₂ ClN ₂ O	307.0625	307.0632	-2.3
						C ₁₈ H ₁₀ ClN ₂	289.0514	289.0527	-4.5
						C ₁₈ H ₁₂ N ₂ O	272.0942	272.0944	-0.7
						C ₆ H ₃ CINO	139.9899	139.9897	1.4
						C ₂ H ₉ ClN ₂ O	112.0398	112.0397	0.9
A	25.4	C ₁₈ H ₁₃ Cl ₂ N ₂ O ₂	359.0337	359.0354	-4.7	C ₁₈ H ₁₂ ClN ₂ O ₂	323.0572	323.0576	-1.2
						C ₆ H ₃ CINO	139.9898	139.9897	-0.7
B	28.3	C ₁₈ H ₁₄ ClN ₂ O	309.0797	309.0795	0.6				
C	31.8	C ₁₈ H ₁₄ ClN ₂ O ₂	325.0757	325.0744	4.0				
F	26.5	C ₁₈ H ₁₃ Cl ₂ N ₂ O ₂	359.0341	359.0354	-3.6	C ₁₈ H ₁₀ ClN ₂	289.0527	289.0527	0
						C ₆ H ₃ CINO	139.9896	139.9897	-0.7
						C ₂ H ₉ ClN ₂ O	112.0398	112.0397	0.9
						C ₁₈ H ₁₂ ClN ₂ O ₂	323.0573	323.0576	-0.9
G	28.2	C ₁₈ H ₁₃ Cl ₂ N ₂ O ₂	359.0338	359.0354	-4.5	C ₆ H ₃ CINO	139.9899	139.9897	1.4
						C ₁₈ H ₁₂ ClN ₂ O ₂	323.0561	323.0576	-4.6
						C ₆ H ₃ CINO	139.9896	139.9897	-0.7
H	29.6	C ₁₈ H ₁₃ Cl ₂ N ₂ O ₂	359.0358	359.0354	1.1	C ₆ H ₃ CINO ₂	155.9842	155.9840	1.3
						C ₅ H ₄ ClN	113.0038	113.0033	4.4
I	24.3	C ₁₈ H ₁₃ Cl ₂ N ₂ O ₃	375.0302	375.0285	4.5	C ₁₈ H ₁₀ ClN ₂ O ₂	321.0436	321.0425	3.4
						C ₆ H ₃ CINO	139.9903	139.9897	4.3

Exp. mass: Experimental exact mass.

Theo. mass: Theoretical exact mass.

were extracted from the TIC of the T24bosc extract, namely *m/z* 325.0757 and *m/z* 309.0797. They correspond to [M+H]⁺ adducts of the empirical formula C₁₈H₁₃ClN₂O₂ (Rt = 31.8 min) and C₁₈H₁₃ClN₂O (Rt = 28.3 min), respectively. The compound C₁₈H₁₃ClN₂O₂ bore only one chlorine atom and one additional oxygen atom compared to boscalid. Most likely, it was correlated to the substitution of one chlorine atom by a hydroxyl function. Two structure proposals existed, but unfortunately, the peak intensity was too low to perform an MS/MS fragmentation. Therefore, these two compounds were synthesized and the retention times of the unknown compounds were compared to standards. This comparison indicated that the chlorine located on benzene ring was substituted by a hydroxyl group (compound C in Fig. 1). The compound C₁₈H₁₃ClN₂O was correlated to a dechlorination of boscalid. As previously stated, the two possible structures were synthesized, and this allowed the identification of a metabolite with the dechlorination occurring on the pyridine ring (compound B in Fig. 1). The second strategy that consisted in the search for the characteristic isotopic pattern of chlorine-containing molecules did not put in evidence other potential metabolites than those identified above.

3.4. Verification of the presence/absence of suspected metabolites in control samples

The presence of all the suspected or identified metabolites was checked in various controls to ensure that they came from the metabolization of boscalid in the honeybee. No signals were detected in the controls, T0bee and T0wat. The analyses put in evidence that the compounds A, G, and H were present in the control T0bosc, corresponding to the sample collected just after boscalid spraying. However the intensities in T0bosc samples were negligible compared to the intensity of the samples after 24 h of metabolization (T24bosc), confirming that the compounds A, G, and H were metabolites of boscalid. As these compounds were absent in the initial spraying solution, their detection in T0bosc can probably be explained by the beginning of the metabolization process during the spraying, in the first moments of contact with the fungicide.

3.5. Analysis of boscalid and its metabolites in collected honeybee samples

Boscalid and the three identified and synthesized substances were analyzed in 37 honeybee samples collected from symptomatic apiaries, i.e., where high bee mortality or abnormal behaviors were observed. The analyses also included the two synthesized substances that were not detected during the *in vivo* experimentation. The results (Table 3) indicate the presence of both boscalid and metabolites/markers of exposure in some of the samples. Among the samples containing boscalid, 8 also contained the hydroxylation product A, in concentrations ranged between 0.2 and 36.3 ng/g. The sample with the highest boscalid content also contained the dechlorinated boscalid product B at the very low level of 0.2 ng/g. To the best of our knowledge, this is the first time that metabolites of boscalid were quantified or even just identified in honeybee samples. The results indicated that the levels of metabolites are not negligible compared to that of the parent molecule and, therefore, contributed to the overall exposure of bees to boscalid. It may be noted that, in several samples, the hydroxylated compound A was quantifiable contrary to boscalid. Moreover, it is worth noting that the compound C, resulting from the substitution of one chlorine atom by a hydroxylated group, was detected once in a sample in which the parent boscalid was not detected. In these cases, the metabolites A and C may be considered exposure markers and may be used to detect and quantify the exposure to boscalid.

4. Conclusion

An original and efficient methodology was developed to generate boscalid metabolites in bees, identify and finally quantify them in environmental samples. This unambiguous identification and quantification, at trace levels, were made possible thanks to the complementary of the mass spectrometric techniques implemented. Thus, several hydroxylation products were identified, including both hydroxylation on the benzene and the pyridine rings. The metabolic pathways also included dechlorination of boscalid and substitution of one chlorine atom by a hydroxyl group. Among the identified compounds, three were quantified in honeybee samples collected from apiaries located in France, at levels

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Table 3

Levels (ng/g) of boscalid and the compounds A, B, C, D and E in honeybee samples from symptomatic hives.

Samples	Boscalid (LOQ 0.1 ng/g)	A (LOQ 0.1 ng/g)	B (LOQ 0.1 ng/g)	C (LOQ 0.1 ng/g)	D (LOQ 0.1 ng/g)	E (LOQ 0.1 ng/g)
1	<LOQ	1.4	nd	nd	nd	nd
2	<LOQ	nd	nd	nd	nd	nd
3	37.5	6.3	nd	nd	nd	nd
4	<LOQ	1.4	nd	nd	nd	nd
5	47.6	36.3	nd	nd	nd	nd
6	<LOQ	2.1	nd	nd	nd	nd
7	<LOQ	1.4	nd	nd	nd	nd
8	<LOQ	1.4	nd	nd	nd	nd
9	<LOQ	nd	nd	nd	nd	nd
10	nd	nd	nd	0.3	nd	nd
11	331.7	27.5	0.2	nd	nd	nd
12–37	nd	nd	nd	nd	nd	nd

nd: not detected.

comparable to or even higher than that of the parent substance. Some of them were quantified even though the parent substance was not quantifiable or not present in the sample. These results indicate or confirm the interest of the scientific community for not solely considering the parent pesticide but metabolites and markers of exposure to the pesticides for toxicological and monitoring studies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.chemosphere.2016.04.135>

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