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METHOD FOR DETERMINATION OF TOTAL IMIDACLOPRID RESIDUES IN SUNFLOWER NECTAR.

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ABSTRACT

The question of toxicity to bees of the insecticide ``Gaucho" has dramatically raised for two years in some production areas of sunflower and honey in France. In the aim of studying the stability and systemic properties of the insecticide in soil and plants, a previously described method for total determination of the ingredient imidacloprid and its metabolites is optimized in the ppb range.
The method is based on the oxidation of the total residues to 6-chloronicotinic acid and determination by gas chromatography linked to tandem mass spectrometry (GC-MS-MS) of the trimethylsilyl ether. Different steps of the methods are simplified and improved to allow analysis on little sized sample (< 0.5 g) with lower detection and quantification limits.

Introduction

Depopulations of beehives were observed for several years in France particularly in some production areas of sunflower. Since this phenomenon was falling in with the increase of the use of the sunflower seed treatment ``gaucho", the insecticide imidacloprid (IMP) was incriminated by beekeepers for toxicity against bees.
Former methods of analysis of imidacloprid used high performance liquid chromatography linked to UV spectrometry (LC-UV) to determine the parent compound or gas-chromatography linked to mass spectrometry (GC-MS) to determine the total residue (Placke and Weber, 1993). These methods were found not sensitive enough because of their quantification limits (LQ) (respectively 20 and 50 ppb) to be used in recent studies. Two years ago, they were then improved by a new technic such as tandem mass spectrometry (MSMS) and many analyses of imidacloprid and total residues were carried out on sunflower parts (leaves, flowers) as well as on bees and honey to study the systemic properties of the insecticide and the transfert to the soil or the insect.

The method based on high performance liquid chromatography linked to tandem mass spectrometry (LC-MSMS) allows to determine imidacloprid and the different metabolites with a 5 ppb LQ except for the olefin metabolite (LQ= 10 ppb). The method based on gas-chromatography (GC) linked to tandem mass spectrometry (MSMS) determine the amount of total residue with a 5 ppbLQ. The GC-MSMS method appears to be cheaper than LC-MSMS because of the lower cost of the apparatus and the single chromatographic analysis needed.
In order to analyse in an inexpensive way and with aQ of 5 ppb samples which size is less than 0.5 g, we developped a method for the analysis of total residue of IMP by GC-MSMS. The method is based on the method described by Placke and Weber (1993), and consists in the extraction and the purification, if necessary, of the total residues, their oxidation to 6-chloronicotinic acid (6-ClNAC), the purification, the silylation of 6-ClNAC and the analysis

by GC-MSMS of the trimethylsilylderivative of 6-ClNAC. The different steps were optimised to allow the treatment of small volumes of solution or extract. In particular, purification on solid phase extraction cartridges, use of an internal standard, and the silylation reaction have been studied and the performances in analysis of nectar are presented and discussed.

Experimental

Standards : 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine (Imidacloprid, IMP) (Cluzeau-Info-Labo, Ste Foy la Grande), 6-Chloronicotinic acid (6-ClNA)(Fluka), 2-Chloronicotinic acid (2-ClNA)(Fluka). Solutions are prepared in acetonitrile at 5 ppb and 50 ppb for IMP ; at 2, 10 and 50 ppb for 6-ClNA ; at 20 ppb for 2-ClNA. Synthetic nectar (glucose 20 %, fructose 20 %, water 60 %).
Reagents : acetonitrile, methanol, hexane (pesticide grade, SDS, Peypin), dry ice, hydrochloric acid solution at 0.01 N, sulfuric acid solution at 10 %, sodium hydroxide solution at 32 %, potassium permenganate solution at 50 g/l, pure water, sodium metabisulfite, phtalic acid solution in acetonitrile at 1g/l, N-methyltrimethylsilyltrifluroacetamide (MSTFA, Macherey-Nagel).
Apparatus : Solid Phase Extraction System (model 10 SPE, Baker) with cartridges Bondelut C18 500 mg/3ml (Varian), Evaporator with nitrogen stream (Reacti-vap, Pierce), 5 ml glass tubes with PTFE screw cap (Chromacol), heating block for 5 ml glass tubes, gas-chromatograph (model 3400, Varian) with a SPI Injector (model 1093, Varian), MSMS detection (Saturn 4D, Varian) fitted with a DB-5 MS column (25 m x 0.25 mm x 0.25 µm) and a deactivated silica retention gap (0.5 m x 0.53 mm), autosampler (model 8200, Varian).

Methods

Purification of residues: 200 µl of 2-ClNA at 20 ppb are tranferred in a 5 ml glass tube and acetonitrile is removed by a nitrogen stream at 40 °C. Then, 200 mg of nectar with 2 ml of HCl 0.01 N are added and the tube is capped and mixed. A C18 Bondelut cartridge is conditionned by percolation of 3 ml of acetonitrile and 2 x 3 ml of HCl 0.01 N. The nectar extract is entirely applied on the C18 cartridge and 2 x 3 ml of HCl 0.01 N and 0.5 ml of acetonitrile are applied on the column for washing. A 5 ml screw cap tube is placed under the cartridge and the purified residues are eluted with 0.5 ml of acetonitrile. Acetonitrile is then removed by a nitrogen stream at 40°C.
Oxidation of residues: 1 ml of pure water, 0.1 ml of NaOH 32 % and 1 ml of KMnO4 50g/l are added in the 5 ml tube which is carefully capped and vigourously mixed. The tube is placed in the heated block at 100 °C during 15 min then cooled in an ice bath to a temperature of about 5 °C. The tube is carefully opened and 1 ml of H2SO4 is added. The tube is cooled again and sodium metabisulfite (100 mg) is added with cooling and stirring until the solution is entirely colourless. The pH is checked (pH<1).
Purification of 6-ClNA and 2ClNA : the aqueous solution is applied on a C18 Bondelut cartridge previously conditionned by percolation of 3 ml of acetonitrile and 2 x 3 ml of HCl 0.01 N. The cartridge is washed with 2 x 3 ml of HCl 0.01 N and 0.5 ml of acetonitrile. A 1.5 ml vial for autosampler is placed under the cartridge and the purified 6-ClNA and 2ClNA are eluted with 0.5 ml of acetonitrile.
Silylation of 6-ClNA and 2ClNA : 50 µl of phtalic acid solution at 1 ppm is added in the 1.5 ml vial and the solvent is removed by a nitrogen stream at 40 °C. After addition of 200µl of MSTFA and 200 µl of hexane, the tube is capped and left at ambiant temperature during one hour before gas chromatography.

Gas-Chromatography : a volume of 2 µl is injected with temperature programming from 60 to 250 °C at 250 °C/min. The column temperature is programmed from 60 to 180 °C at 15 °C/min and from 180 to 300 °C at 30 °C/min. The tranfer line temperature is 260 °C.
Tandem Mass Spectrometry : the ion trap temperature is set at 210 °C. The parent ion is isolated at m/z = 214 for both 6-ClNA and 2-ClNA. The daughter ion are produced by the non resonant excitation mode (V_{CID} = 57 V and Qz = 0.3) at m/z 170 for 6-ClNA and m/z=178 for 2-ClNA.
Calibration : The quantification is realised by internal calibration. The response of 6-ClNA versus 2-ClNA is determined in a range of concentration from 0 vs 20 ppb to 50 vs 20 ppb.

Results and discussion

Use of an internal standard : 2-ClNA was chosen because of its structural analogy with 6-ClNA necessary for the MSTFA derivatization and GC-MSMS analysis. So, the same ion (m/z 214) could be isolated and fragmented in the same conditions for 2-ClNA and 6-ClNA. The quantification was then specificaly realized with respectively ions m/z 178 and 170.
The benefit of using an internal standard generally appears when losses of extract or sample occur during the analysis. This is the case with the microscale method because a small loss of analyte often represents a significant decrease of the recovery yield. Table 1 shows that althought the recovery yield for 2-ClNA and 6-ClNA are less than 70%, the recovery yield for the amount of 6-ClNA calculated with the internal standard is near 90 %.

Table 1 : Recovery of 6-ClNA and 2-ClNA 0.2 ml solutions after purification on C18 Bondelut cartridges. (Cartrigdes were previously conditionned with 3 ml acetonitrile and 2 x 3 ml HCl 0.01 N)

Concentration of the 0.2 ml solutions	Yield of 6-ClNA	Yield of 2-ClNA	Quantification of 6-ClNA/2-ClNA	Yield of 6-ClNA/2-ClNA
100 ppb	60.1	57.3	105 ppb	104.9 %
20 ppb	67.0 %	69.2 %	19.3 ppb	96.8 %
5 ppb	63.0 %	68.0 %	4.63 ppb	92.6 %

Purification on C18 cartridges : For nectar analysis, purification is needed to remove the high content of sugars (glucose and fructose) before the oxidation step. Due to its hydrophobic properties, IMP can be easily separated from the hydrophilic sugars (in LC analysis of IMP on a C18 stationary phase, the acetonitrile amount in the eluent have to be higher than 25 %). In opposite, acido-basic properties of 6-ClNA and 2-ClNA allow quantitative linkage on C18 only in well defined conditions. The two molecules and particularly 2-ClNA need a pH value less than 2 to become non-ionic and then, hydrophobic enough to be retained on the C18 phase. The solution containing 6-ClNA and 2-ClNA applied on C18 and the washing solution must be acidified by a strong and volatile acid to assure a low pH and to be removed before the silylation step. The elution of the two molecules is very easy with two fractions of 0.5 ml of acetonitrile. Table 1 show some results on purification experiments carried out on standard solutions of 6-ClNA and 2-ClNA at 100, 20 and 5 ppb

Oxidation of residues : oxidation of IMP and metabolites is known as a critical step due to the instability of 6-ClNA in basic medium. Moreover, when realized in large vessels (250 ml) under reflux the control of the reaction is tricky and the conditions are inappropriate to the little size of the nectar sample. Micro-scale oxidation in sealed tube appears to be a noticeable

improvement since the ability to quickly cool the tube in an ice bath at the end of the reaction may prevent the 6-ClNA breakdown. Kinetic of oxidation in capped tubes was investigated and showed that the yield was at 100 °C complete and stable from 10 to 20 min. The micro-scale oxidation leads to a significant simplification and safety in the operating procedure.

Silylation and GC-MSMS: The former procedure of silylation 6-ClNA used acetonitrile and MSTFA (Placke and Weber, 1993). Our first experiments on 6-ClNA analysis GC-MSMS failed because of the reactivity of the stationary phase with acetonitrile and MSTFA. The problem was solved with the replacement of acetonitrile by hexane, the high polarity of acetonitrile being responsible for the non-reversible adsorption of silylated 6-ClNA. A side effect of the change of the solvent was the increase of the response by 5 . The detection and the quantification limit were respectively 0.5 and 1 ppb when 2 µl of solution were injected.

Analysis of nectar : Our first experiments indicate that it is possible to detect less than 1 ng of imidaclopride corresponding to a concentration of 5 ppb in a 0.2 g sample.

Conclusion

The optimisation of the method was necessary to analyse the nectar which is available only in low quantities. The new microscale procedure is significantly more useful and safety because of the non-use of solvents and time consumming operation. First results about the LD and the LQ are promising considering the low weight of the sample analysed. Work is in progress to improve LD, LQ and to validate the method in agreement with the Good Laboratory Practice and the Codex Alimentarius specifications. Further work will concern the opimisation of the method for analysing imidacloprid in other complex matrix.

References

Placke F.-J. and Weber E., 1993. Method of determining imidacloprid residues in plant materials. *Planzenschutz-Nachrichten Bayer*, **46**, 109-182.