Trace-level determination of two neonicotinoid insecticide residues in honey bee royal jelly using ultra-sound assisted salting-out liquid liquid extraction followed by ultra-high-performance liquid chromatography-tandem mass spectrometry

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TRACE-LEVEL DETERMINATION OF TWO NEONICOTINOID INSECTICIDE RESIDUES IN HONEY BEE ROYAL JELLY USING ULTRA-SOUND ASSISTED SALTING-OUT LIQUID LIQUID EXTRACTION FOLLOWED BY ULTRA-HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

A few studies have investigated the analysis of neonicotinoid insecticides in honey bee royal jelly. An analytical method based on salting-out liquid-liquid extraction assisted with ultrasound followed by liquid chromatography-tandem mass spectrometry was developed for the determination of thiamethoxam and clothianidin in royal jelly. A cleaning step based on freezing before injection to the UHPLC-MS/MS (MRM mode) allowed maximizing the signal-to-noise ratio. The finalized method was validated in line with European legislation. The limits of quantification (LOQ), reached from a test sample of 100 mg royal jelly only, was 0.25 ng/g for both neonicotinoids. The recoveries were close to 100% with relative standard deviation below 13.7%, measured at three concentration levels. The validated method was finally applied to samples of royal jelly commercially available, and showed the presence of thiamethoxam at levels between the limit of determination (LOD = 0.15 ng/g) and LOQ in a few samples.

Keywords: thiamethoxam; clothianidin; QuEChERS; traces; LC-MS/MS

1. INTRODUCTION

Neonicotinoids represent the most widely used insecticides in the world [1]. By acting selectively on insect nicotinic acetylcholine receptors, they control a broad spectrum of insect pests [2]. Due to their systemic action, they may translocate throughout the plants to the pollen or nectar, which may result in exposure to foraging bees. Their potentially adverse or toxic effects on non-targeted organisms, including honey bees or bumble bees, have been widely demonstrated and described in the literature [3, 4, 5]. As a consequence, the European Union decided to restrict their use on field crops in 2018 [6].

Many studies have investigated the effects of neonicotinoids on worker honey bees that are essential to the functioning and maintenance of the colony. The queen can however be considered as the most important individual of the colony since she is the sole responsible for egg laying as well as the source of colony cohesion via its pheromones [7]. The preservation of the queen's good health is a determining factor in ensuring the survival of bee colonies. Recent studies have focused on the effects of neonicotinoids on queens health/behaviour [8-12].

Thiamethoxam and clothianidin (clothianidin is an active substance in insecticidal formulations and also the most toxic metabolite of thiamethoxam) are two such neonicotinoids that have been shown to negatively affect queens [8, 9]. Short- and long-term effects on wild or domestic queens suggest that both neonicotinoids may contribute to colony weakening in different ways. For example, it has been observed that *A. mellifera* queens exposed for four-weeks to field realistic concentrations of thiamethoxam and clothianidin (4 and 1 ng/g, respectively) has serious implications for their reproductive anatomy and physiology [9]. More recent studies have confirmed adverse effects on fertility, or the reduction of the average terminal oocyte length in several species of wild bumblebee queens [10, 11]. A diet enriched with clothianidin (5-10 ng/g) affected the survival of *Bombus impatiens* queens after a short period of 7 days [12]. Furthermore, consuming clothianidin over a longer period of time had a negative impact on wild bumblebee population dynamics by decreasing reproductive capacity, reducing the number of individuals mating in the fall and the number of queens nesting in the spring [12]. A reduction in the diet of queens of several wild bumblebees was observed in the presence of low doses of thiamethoxam (1.87-5.32 ng/g) which may suggest an anti-food, repellent or toxic effects [10].

Royal jelly is the most elaborate substance in the hive, a highly sugary and proteinaceous secretion produced by the hypopharyngeal and mandibular glands of workers. As its name

suggests, it is mainly intended for feeding developing queens during their first days of development. Royal jelly allows the rapid and important growth of queen larvae and allows the queen to lay a large quantity of eggs. To better understand the role of thiamethoxam and clothianidin on queen development, and also to assess the amount of pesticides with which developing queens are likely to be exposed, it is essential to measure potential neonicotinoid levels in royal jelly. It is therefore important to have an analytical method that is sufficiently efficient to characterize traces of thiamethoxam and clothianidin residues in royal jelly. Composed of carbohydrates, proteins, lipids, minerals and vitamins, royal jelly is a complex matrix. Moreover, as honey bees only produce the quantity necessary for brood rearing, it is not stored and can therefore be taken only in very limited mass from the hive. The development of a sensitive method from a small quantity of royal jelly is therefore an analytical challenge.

A very few studies have investigated the analysis of neonicotinoid insecticides in royal jelly. To our knowledge, only three methods have been proposed, including thiamethoxam and clothianidin, to characterize the presence of pesticides in royal jelly intended for consumption as fresh jelly or as a liquid preparation [13, 14,15]; each had unique protocols. The sample preparation method, that includes the extraction of the target compound and the clean-up, is a key step in the analysis of royal jelly. Martinez-Dominguez and coauthors [13] preferred the "dilute and shoot" to the QuEChERS method (acronym of quick, easy, cheap, effective, rugged and safe), which is widely used for the analysis of pesticides in food or environmental matrices. Indeed, QuEChERS did not provide satisfactory results in terms of recoveries for all the targeted molecules (260 compounds including only a few neonicotinoids). The extraction was performed with acidified acetonitrile, and was followed by a clean-up with anhydrous zirconium oxide. Combined with ultra-high performance liquid chromatography (UHPLC), and then high resolution mass spectrometry (HRMS) with an Orbitrap, the protocol only achieved limits of quantification (LOQ) of 10 ng/g for thiamethoxam and clothianidin. The analytical work-flow was however perfectly adapted to the wide screening of substances in royal jelly, and the an initial test sample was relatively high (2 g). Additionally, Valverde eand coauthors [14] evaluated different types of neonicotinoid extraction in royal jelly-based products. Solid phase extraction (SPE) was first considered, but eventually given up due to the viscosity of the sample under loading conditions (ammonium formate based) as this caused the cartridge to clog. Tests with the QuEChERS method have also proved unsatisfactory results because of the appearance of an interface that hinders solvent recovery

and thus affects the reproducibility of the results. Finally, the proposed alternative was dispersive liquid-liquid microextraction followed by a UHPLC analysis coupled with HRMS (QToF). The proposed method was based on a low test sample (100 mg) exhibited LOQ of 6 and 7 ng/g, respectively, for thiamethoxam and clothianidin, which is insufficient in a context of apiarian queen contamination via royal jelly [9, 12]. Hou and coauthors [15], for their part, developed an effective royal jelly clean-up method based on SPE, followed by HPLC analysis coupled with tandem mass spectrometry (LC-MS/MS) with multiple reaction monitoring (MRM) mode. Their protocol allowed an LOQ of 0.5 ng/g for thiamethoxam and clothianidin to be reached, but required an initial mass of 2 g of royal jelly.

The objective of this work was to develop a specific method for the analysis of thiamethoxam and clothianidin in honey bee royal jelly in order to lower both the test sample mass and the current LOQ. Taking into account previous work using the QuEChERS technique, or a modification [16, 17, 18], for the quantification of pesticide traces in beekeeping matrices, we started from a liquid-liquid extraction assisted by salts. We then sought to optimize the method so that it was both sensitive and simple. Next, we developed an efficient extraction protocol that limits matrix effects. This was based on a salting-out liquid-liquid extraction, close to the first step of the QuEChERS method, assisted by ultrasound. A cleaning step was based on freezing, before injection by UHPLC-MS/MS (MRM mode) which was optimized to maximize the signal-to-noise ratio (S/N). The finalized method was validated in line with European legislation dealing with analytical quality control and method validation procedures for pesticide residues [19] and then applied to samples of royal jelly commercially available.

2. MATERIALS AND METHODS

2.1 Standards and reagents

Analytical standards of thiamethoxam, clothianidin, thiamethoxam-d3, clothianidin-d3 and imidacloprid-d4 were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France). All standards were > 97% purity. Individual stock solutions were prepared at concentrations of 1000 µg/mL in methanol (MeOH) and were stored at -23 °C during six months. Working solutions were prepared daily by appropriate dilution of an intermediate stock solution (10 µg/mL of each compound in MeOH stored at -23 °C). An internal standard mixture solution containing thiamethoxam-d3 and clothianidin-d3 at 100 ng/mL in MeOH was prepared. A solution of imidacloprid-d4 at 100 pg/mL in water/MeOH (80/20, v/v) was used as injection

standard. Acetonitrile (ACN) UPLC-MS grade, MeOH UPLC-MS grade and n-heptane HPLC-S grade were obtained from Biosolve Chimie (Dieuze, France). Water (Optima quality) was furnished by Fischer Scientific (Illkirch, France). Ammonium hydroxide solution (NH_3, aq ; 25% in water) was obtained from Sigma-Aldrich. Buffer extraction packets were obtained from Agilent Technologies (Les Ulis, France). The acetate buffer (AOAC method) contained 1.5 g of sodium acetate (CH_3COONa) and 6 g of magnesium sulfate ($MgSO_4$), while the citrate buffer (EN method) contained 1 g of sodium citrate ($Na_3C_6H_5O_7$), 4 g of $MgSO_4$, 1 g of sodium chloride (NaCl) and 0.5 g of sodium hydrogencitrate sesquihydrate ($HOC(COOH)(CH_2COONa)_2 \bullet {}_1/_2H_2O$). The appropriate amount of buffer was weighed from these packets. The dispersive PSE phases PSA (primary and secondary amine exchange, containing 0.9 g $MgSO_4$ and 0.15 g of Chromabond® diamino) and PSA-C18 (containing 0.9 g $MgSO_4$, 0.15 g of Chromabond® diamino and 0.15 g of Chromabond® C18) were obtained from Macherey-Nagel (Hoerdt, France).

2.2 Sampling procedure

Royal jelly from an organic producer “La grange aux apiculteurs” (Lapenne, France) considered to be blank (i.e. free of any pesticides) was used for development and validation phases. During the study, the absence of contamination of this matrix of reference by the target compounds was verified. It was also used to prepare the calibration curve when quantifying the samples collected in the field.

2.3 A Salting-out microextraction

After defrosting the royal jelly, a 100 mg aliquot was weighted in a 2 mL polypropylene centrifuge tube containing 3 zirconium balls (3 mm internal diameter). A volume of 50 µL of internal standard solution was added and the tube was centrifuged again at 10,000 rpm for 3 minutes before being placed in a vacuum concentrator (MiVac, Genavac) at 30 °C for 30 minutes. The salting-out microextraction was performed by adding 100 µL of water then shaken with a vortex mixer for 20 s. Next, 1 mL ACN was introduced into the tube, shaken with a vortex for 20 s, transferred to a ultrasonic bath (Branson, 5510 model) for 5 min at 40 kHz, and finally, 300 mg of acetate buffer, was added to the mixture. The tube was then introduced into BeadBug microtube homogeneizer (Benchmark Scientific, Sayreville, USA), at 3,200 rpm for 2 min. Next, the tube was centrifuged at 10,000 rpm for 5 min. A volume of 750 µL of the supernatant was transferred to a 2 mL vial and incubated for 15 h at -18 °C.

Afterwards, 500 µL volume of the supernatant was shifted to another 2 mL vial and evaporated to dryness in the vaccum concentrator at 30 °C. Subsequently, the dry residue was dissolved in this vial in same volume of water/MeOH (80/20, v/v) injection standard for the UHPLC-ESI-MS/MS analysis.

2.4 UPLC-ESI-MS/MS analysis

Liquid chromatography was performed using an H-Class UPLC system from Waters (Saint Quentin en Yvelines, France). Various chromatographic columns were evaluated: Kinetex Phenyl-Hexyl (50 x 2.1 mm; 2.6 µm) and Kinetex C18 (50 x 2.1 mm; 2.6 µm) from Phenomenex (Le Pecq, France), Poroshell 120 Phenyl-Hexyl (50 x 3 mm; 2.6 µm) and Poroshell EC C18 (50 x 2.1 mm; 1.9 µm) from Agilent Technologies. Lastly, the separation was performed with a Kinetex Phenyl-Hexyl (50 x 2,1 mm; 2,6 µm) with a mobile phase composed of (A) 0.02% NH_4OH in water and (B) MeOH with the following gradient: 5-90% (B) for 1.7 min followed by 100% (B) for 1.2 min. The column was then equilibrated at the initial conditions for 2 min. The flow rate was 0.4 mL/min, the oven temperature was 40 °C and the injection volume was 3 µL.

The chromatographic system was coupled to a Xevo-TQ-S triple quadrupole mass spectrometer from Waters, equipped with a StepWave ion guide to enhance sensitivity. Electrospray ionisation was performed in the positive mode with the following parameters: capillary voltage 3200 V, desolvatation temperature 550 °C, source temperature 150 °C, and nitrogen desolvatation and nebuliser gas flow 900 L/H and 150 L/H, respectively. For each compound, Intellistart™ (Waters) software was used to automatically select the *m/z* value for the precursor ion as well as product ion, cone voltage and collision energy. Thus the protonated molecular ions $[M+H]^+$ were selected as the precursor ions. Two multiple reaction monitoring (MRM) transitions were optimized: the target ion transition with the highest intensity (MRM1) was used for quantification, whereas the second target ion transition (MRM2) was used for confirmation. The ion transitions, cone voltages, collision energies and dwell times are collected in Table 1.

Table 1: Transitions used for the quantification (MRM1) and confirmation (MRM2), dwell time and source parameters

Compound	Transitions	Dwell times (ms)	Cone voltage (V)	Collision energy (eV)
Thiamethoxam	MRM1: 292.0 > 211.0	3	10	12
	MRM2: 292.0 > 181.0	3	10	12
Clothianidin	MRM1: 250.0 > 168.9	3	20	14
	MRM2: 250.0 > 132.0	3	20	16
Thiamethoxam-d3	MRM: 295.0 > 214.0	3	12	10
Clothianidin-d3	MRM: 253.0 > 172.0	3	22	12
Imidacloprid-d4	MRM: 260.2 > 213.2	3	24	16

2.5 Method validation

Validation of the method was based on the SANTE/11813/2017 guidance document [19]. The linearity, matrix effect (ME), limits of determination (LOD) and LOQ, specificity, precision and recovery were evaluated in blank matrix spiked with standards of thiamethoxam and clothianidin. Linearity was evaluated from unspiked royal jelly and six spiking points between LOQ and 30 LOQ (i.e. between 0.25 and 7.5 ng/g). Calibration curves (n = 7) were constructed by plotting the analyte peak area against the analyte concentration. The study of the linearity was repeated four times. The (ME), which reflects the extent to which the matrix modifies the ESI-MS/MS signal, was evaluated by comparing the response of a sample of royal jelly that underwent the extraction protocol and was then spiked with a standard solution in the solvent at the same concentration. The (ME) was evaluated at three different concentrations (n=6). LOQ was defined as the lowest spike level meeting the method performance criteria in terms of trueness and precision. It was evaluated by considering the MRM1 transition. The LOD was defined as the lowest concentration that can be reliably reported with the method. It was evaluated as the concentration for which S/N of three was observed considering the MRM2 transition.

The identification of the analytes was guaranteed by both chromatography and tandem mass spectrometry. Thus, the compounds were identified by their retention time (correspondence ± 0.1 min with matrix-matched standard), the presence of the characteristic MRM1 and MRM2 transitions and the ion ratio being within ± 30% of matrix-matched standard from the same sequence). Specificity was verified by comparing the injections of extracts of unspiked royal jelly and spiked royal jelly. Recoveries were obtained by spiking blank royal jelly at three

concentration levels (0.25, 0.5 and 2.5 ng/g). The peak area of the spiked royal jelly extract before sample preparation (n=6) was compared to the peak area of the royal jelly extract that underwent the sample preparation protocol and was spiked afterwards. The intra-day precision was deduced from these six replicates for three different concentrations.

3. RESULTS AND DISCUSSION

3.1. Optimisation of LC-MS/MS conditions

The chromatographic conditions were first optimized with respect to the choice of the column, the mobile phase composition and the temperature of injection by injecting 5 µl of a mixture of the 2 compounds at 1 µg/L in water/MeOH mixture (90/10, v/v). As a starting point for optimizing chromatographic parameters, we compared two types of columns based on C18 and phenyl-hexyl phases, respectively. C18 columns have been the most commonly used for environmental analyses and have shown their effectiveness for the analysis of neonicotinoids in apiarian matrices [20, 15, 16]. On the other hand, previous developments in the laboratory [16] have shown that phenyl-hexyl is also interesting for the separation of insecticides including neonicotinoids. The first tests were carried out under isocratic conditions with the mobile phases that we had optimized for the simultaneous analysis of pyrethroids and neonicotinoids in beeswax [16] (i.e. with an aqueous phase composed of ammonium acetate and acetic acid (75%)), and MeOH as the organic phase (25%). As a selection criterion, we considered a short separation time and especially the highest S/N. We worked with short columns of 50 mm. We have only selected columns filled with superficially porous particles (fused-core technology) because they exhibit the advantages of porous particles (sufficient loading and retention capacity) and non-porous particles in terms of kinetic performance. They therefore allow a gain in speed, resolution and sensitivity, while maintaining an efficiency close to porous sub-2µm particles.

Under these isocratic conditions, a slightly lower resolution appears with columns based on a phenyl-hexyl phase, designed to promote retention and separation of aromatic compounds, but the S/N is in favour of this type of column (Figure 1). We evaluated two pheny-hexyl based columns from two different processes for manufacturing the particles: the sol-gel technique for the Kinetex column and the one-step polymerization technique for the Poroshell column. The Kinetex Phenyl-Hexyl column was finally selected for further optimization,

because retention times were shorter, and the S/N of the thiamethoxam peak was 20% higher with this column (Figure 1).

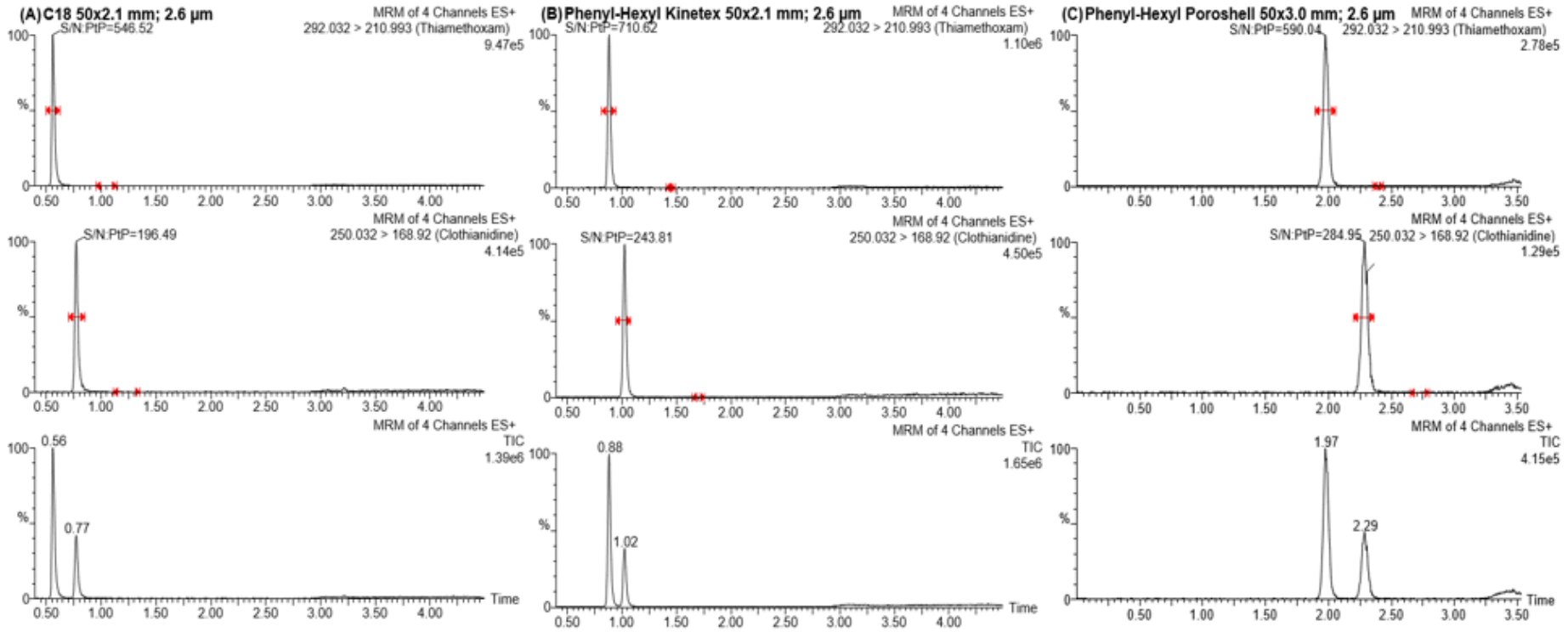

Figure 1: Comparison of the separation of clothianidin and thiamethoxam as well as S/N on different types of chromatographic columns.

The desolvation temperature and capillary voltage have been adjusted. An increase in temperature significantly increased the signal of thiamethoxam, while that of clothianidin was only slightly affected (Figure 2A). Similarly, an increase in capillary voltage exhibited a positive impact on the detection of thiamethoxam, with a 70% increase in signal between 2.5 and 3.2 kV (Figure 2B). These parameters were finally set at 550 °C and 3.2 kV, respectively, that corresponded to values slightly lower than the maximum temperatures and voltages recommended for the device.

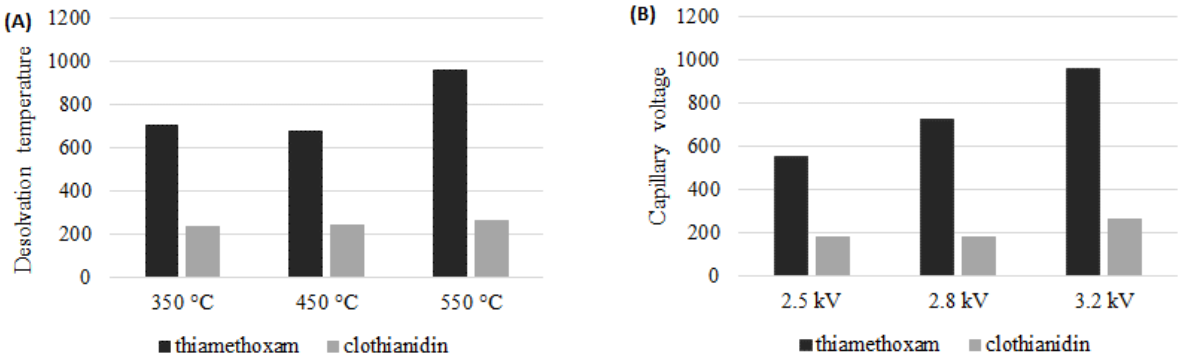

Figure 2 : Influence of desolvatation temperature (A) and capillary voltage (B) on the signal of thiamethoxam and clothianidin.

To improve the signal response by MS/MS, mobile phase modifiers such as ammonium acetate, formic and acetic acid, and ammonium hydroxide were also considered. The mobile phases to maximize the S/N were based on the addition of NH_4OH in the mobile phase. The elution gradient was adjusted to retain the one presented in section 2.4. Finally, the column temperature was studied (40, 50 and 60 °C). As expected, the retention time increased as the temperature decreased. On the other hand, the S/N increases inversely with temperature. Thus, between 60 and 40 °C an increase of 20% and 30% was observed for the thiamethoxam and clothianidin signals, respectively.

3.2. Sample preparation protocol

We carried out first tests of salting-out assisted acetonitrile extraction (with 500 mg acetate buffer) from 100 mg royal jelly. We added 500 µL of water to the 500 µL of acetonitrile to approximate the proportions recommended by Anastassiades and coauthors [21] for the first stage of the QuEChERS extraction. This preliminary test was conclusive since recoveries close to 100% were observed for both molecules, with LOQs of 1.4 and 2.9 ng/g for

thiamethoxam and clothianidin, respectively, being obtained. Unlike a previous study [14], no disturbing interface formation was observed, which convinced us to continue the optimization of this technique for extraction. For the rest of the development, we sought to reduce the LOQ, while keeping the method as simple as possible.

The proportion of water during extraction is an important parameter. We observed that as the proportion of water decreases, the signal increases. Thus, the areas were improved by a factor of 4 when the volume of water decreased from 500 µL to 100 µL. We therefore maintained a proportion of water of 100 µL during extraction, which corresponded to the minimum quantity to ensure the dissolution of royal jelly. It should be mentioned, however, that with such a small volume of water, royal jelly agglomerated after acetonitrile removal; this limited us to one solvent extraction.

The second step of the classic QuEChERS method is a dispersive phase purification (dSPE). This step is important because it eliminates some of the impurities that represent analytical interferences that can inhibit the signal (the so called matrix effects - ME). It thus contributes to the lowering of the LOQ while often providing robustness to the method. However, it is difficult to optimize because the extraction efficiency must not be degraded by this additional step. The benefits of the two dispersive phases PSA and PSA/C18 were evaluated to improve the signal, considering the S/N. Since royal jelly is partly made up of lipids (4.5 %), we also studied the influence of adding a small volume of heptane to improve clean-up.

Overall, the S/N were improved by clean-up, especially for thiamethoxam. The use of the PSA phase improved the S/N of both neonicotinoids (by a factor of about 4 and 21, for thiamethoxam and clothianidin, respectively) compared to extraction conducted without additional clean-up (Figure 3). The corresponding LOQ were 0.1 ng/g and 0.4 ng/g for thiamethoxam and clothianidin, respectively. The clean-up with heptane, alone or in combination with a PSA phase, greatly increased the thiamethoxam signal but did not provide a significant improvement for the clothianidin.

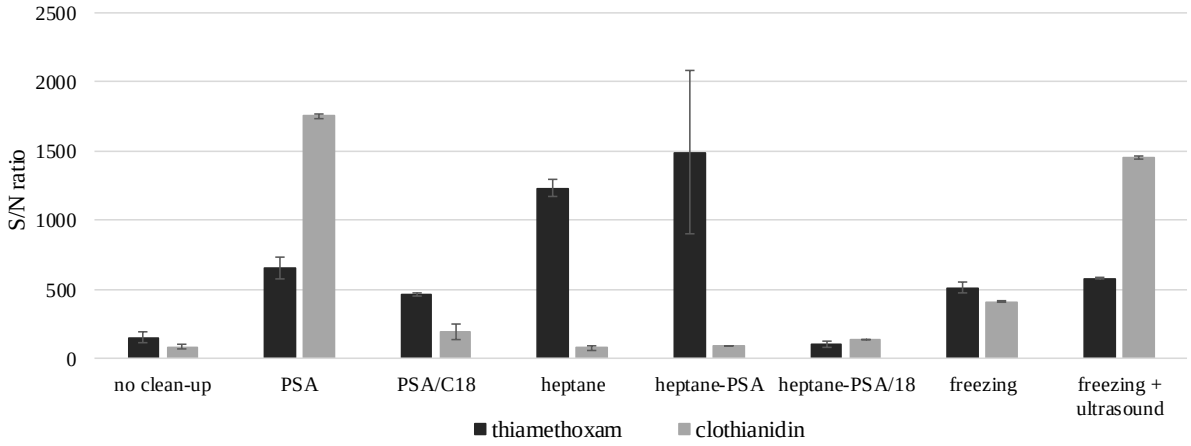

Figure 3: Influence of the clean-up step on the S/N ratio of thiamethoxam and clothianidin: 150 mg PSA, 150 mg PSA/C18, 150 µL heptan, ultrasounds, freezing (n=2).

The step of clean up using a dispersive phase was relatively meticulous and repetitive (weighing the dispersive powder phase, risk of loss or contamination during handling), we explored the alternative of freezing. For a complex hive product such as bee bread, we had previously shown that this step promotes the precipitation of interfering compounds (sugar or lipid type), thus allowing their elimination [16]. Figure 3 shows that this freezing improves the S/N compared to extraction without clean-up, but does not reach the S/N obtained with the use of the PSA dispersive phase, especially in the case of clothianidin. Always with the idea of improving the S/N while avoiding the use of a clean-up based on an adsorbent phase, we added a step in an ultrasound bath during extraction. We observed an improvement in the S/N, close to the values obtained with the dSPE (Figure 3). There was also an improvement in the aspect of the extracts, which appear more homogeneous from one extraction to another, when using ultrasound. This resulted in improved repeatability of the whole protocol.

3.3. Validation of the method

The whole method, including the sample preparation and the UHPLC-MS/MS analysis, was validated in terms of specificity, linearity, ME, precision and recoveries, LOD and LOQ (Table 2). The use of the MRM mode ensured the specificity of the method. This was verified by extracting (n=6) organic royal jelly *a priori* free of pesticides. We did not observe interfering signals with a retention time close to the two compounds of interest. As expected with the use of an electrospray ionization source, ME was observed, affecting the ion signal in the royal jelly extract compared to an analysis in the solvent alone. The quantification of the two neonicotinoids should therefore be performed using matrix-matched calibration curves.

The linearity of these curves (n=4) was studied between LOQ and 30×LOQ (i.e. between 0.25 and 7.5 ng/g). It was evaluated by the calculation of the determination coefficients (R^2) and by a retrospective calculation of the concentration of each of the six individual calibration standards (the concentration values of the standards of one curve were evaluated from the other three calibration curves). In order to avoid possible differences between the royal jellies analyzed, an additional verification with a deuterated standard was added. This supplementary precaution was justified considering the very low levels of concentration targeted. The R^2 values were all superior to 0.992, and the recalculated values of the standards did not deviate by more than 5% from the expected value. The recoveries of the spiked samples ranged between 94.6 and 104.3%, indicating good recovery of the method. A satisfactory precision was obtained since the relative standard deviations (RSDs) values of royal jelly were comprised between 5.3% and 13.7% for the 2 compounds at 3 concentration levels. The LOD and LOQ were very low (0.15 and 0.25 ng/g, respectively), and inferior to those previously reported in the literature for these two molecules. The method developed therefore offers the possibility of characterizing ultra-traces of thiamethoxam and clothianidin from a very limited quantity of royal jelly.

Table 2: LOD and LOQ, mean recoveries, intermediate precisions and matrix effects obtained from spiked royal jelly

Compound	LOQ ng/g	LOD ng/g	Recovery mean (%)			Intermediate precision (n=6) %RSD			Matrix effect mean (%) (%RSD, n=6)		
			Low	Medium	High	Low	Medium	High	Low	Medium	High
Thiamethoxam	0.25	0.15	104.3	98.5	94.6	13.7	9.0	8.4	79 (12)	82 (3)	85 (3)
Clothianidin	0.25	0.15	99.2	102.9	95.2	12.7	13.4	5.3	81 (5)	80 (5)	80 (3)

3.4. Application of the method

The method thus developed and validated was applied to determine potential residues of thiamethoxam and clothianidin in 11 royal jelly samples available from an online shopping site. Each sample was extracted in duplicate, and each extract was injected twice. Clothianidin was never detected in these samples. However, thiamethoxam was detected in three of them, with a concentration comprised between LOD and LOQ, i.e. between 0.15 and 0.25 ng/g (Figure 4). It is important to note that the conditions under which samples were taken or stored (time to packaging, temperature control, freezing, thawing, etc.) were not known. It is reasonable to assume that the method could be useful for assessing neonicotinoid levels in freshly collected jellies from apiaries.

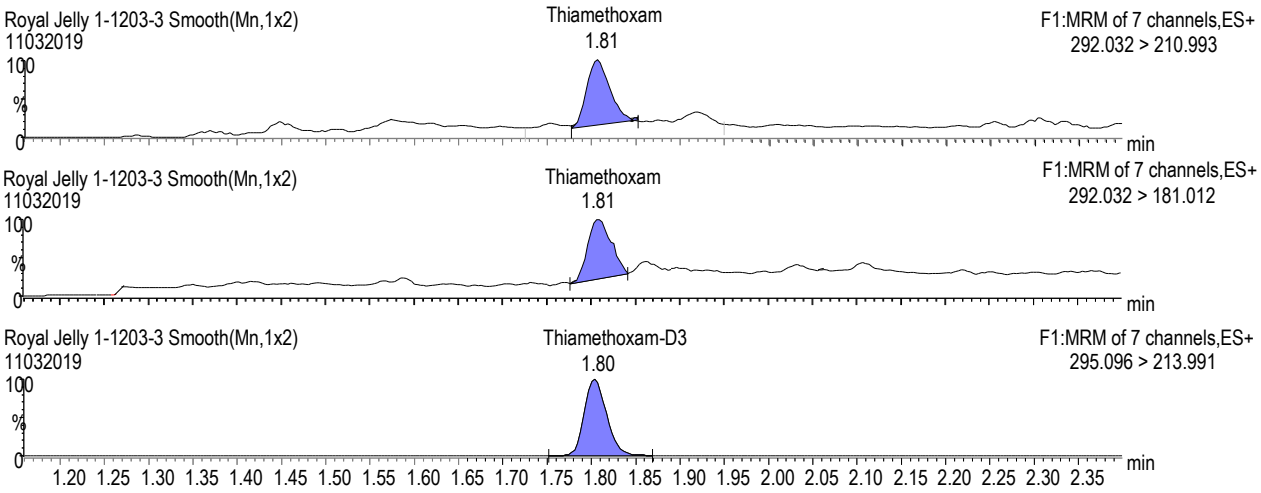

Figure 4: Example of a royal jelly in which thiamethoxam was detected (top to bottom: MRM1 transition of thiamethoxam, MRM2 transition of thiamethoxam and MRM transition of deuterated thiamethoxam used as internal standard).

5. CONCLUSIONS

In the present study, the new method combines an efficient sample preparation protocol with UPLC-MS/MS for the determination of traces of thiamethoxam and clothianidin in royal jelly. This method is interesting because it allows to reach ultra-trace levels (LOD 0.15 ng/g; LOQ 0.25 ng/g) from a test sample of only 100 mg, which was not possible until now. These performances make it possible to use the method for assessing neonicotinoid levels in freshly collected royal jelly collected from honey bee colonies.

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