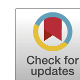
Environmental Pollution 247 (2019) 964–972

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Ultra-trace level determination of neonicotinoids in honey as a tool for assessing environmental contamination☆



Souad Kammoun [a], Blaise Mulhauser [b], Alexandre Aebi [c, d], Edward A.D. Mitchell [c], Gaétan Glauser [a, *]

[a] Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Avenue de Bellevaux 51, 2000, Neuchâtel, Switzerland
[b] Botanical Garden of Neuchâtel, Chemin du Pertuis-du-Sault 58, 2000, Neuchâtel, Switzerland
[c] Laboratory of Soil Biodiversity, University of Neuchâtel, Rue Emile-Argand 11, 2000, Neuchâtel, Switzerland
[d] Anthropology Institute, University of Neuchâtel, Rue Saint-Nicolas 4, 2000, Neuchâtel, Switzerland

A R T I C L E I N F O

Article history:
Received 17 December 2018
Received in revised form
31 January 2019
Accepted 2 February 2019
Available online 5 February 2019

Keywords:
Systemic insecticides
Sulfoxaflor
Flupyradifurone
UHPLC-MS/MS
QuEChERS

A B S T R A C T

Neonicotinoids and the closely related insecticide classes sulfoximines and butenolides have recently attracted growing concerns regarding their potential negative effects on non-target organisms, including pollinators such as bees. Indeed, it is becoming increasingly clear that these effects may occur at much lower levels than those considered to be safe for humans. To properly assess the ecological and environmental risks posed by neonicotinoids, appropriate sampling and analytical procedures are needed. Here, we used honey as reliable environmental sampler and developed an unprecedentedly sensitive method based on QuEChERS and UHPLC-MS/MS for the simultaneous determination of the nine neonicotinoids and related molecules currently present on the market (acetamiprid, clothianidin, dinotefuran, flupyradifurone, imidacloprid, nitenpyram, sulfoxaflor, thiacloprid and thiamethoxam). The method was validated and provided excellent levels of precision and accuracy over a wide concentration range of 3–4 orders of magnitude. Lowest limits of quantification (LLOQs) as low as 2–20 pg/g of honey depending on the analytes were reached. The method was then applied to the analysis of 36 honey samples from various regions of the World which had already been analysed for the five most common neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) in a previous study. This allowed us to determine the long-term stability (i.e. up to 40 months) of these molecules in honey, both at room temperature and −20 °C. We found that the five pesticides were stable over a period of several years at −20 °C, but that acetamiprid and thiacloprid partially degraded at room temperature. Finally, we also measured the levels of dinotefuran, nitenpyram, sulfoxaflor and flupyradifurone and found that 28% of the samples were contaminated by at least one of these pesticides.

© 2019 Elsevier Ltd. All rights reserved.

1. Introduction

Since their introduction in 1990, neonicotinoids have become the most widely used insecticides in terms of global market share, replacing older pesticides such as organophosphates and carbamates (Jeschke et al., 2011). Neonicotinoids can be seen as a group of structurally diverse insecticides that share a common mechanism of action, i.e. they act as agonists of nicotinic acetylcholine receptors (Simon-Delso et al., 2015). They are called systemic insecticides, being transferred after administration to all plant organs and thus efficiently protecting plants from herbivorous insects (Jeschke et al., 2011; Bonmatin et al., 2015). Moreover, they are highly persistent in the environment, with half-lives of several months or even years under natural conditions (Bonmatin et al., 2015). As a consequence, non-target insects, in particular pollinators such as bees, and other invertebrates and vertebrates are highly prone to risks of exposure to neonicotinoids (Goulson, 2013). While generally not directly lethal at field-realistic concentrations, neonicotinoids may adversely affect the health of bees and other non-target insects by inducing so-called sub-lethal effects, such as neurological and cognitive disorders, reduced foraging and homing capacity, immune suppression, or reproduction efficiency (Sánchez-Bayo et al., 2016; Pisa et al., 2017). These sub-lethal effects

☆ This paper has been recommended for acceptance by Charles Wong.

* Corresponding author. Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Avenue de Bellevaux 51, 2000, Neuchâtel, Switzerland.
E-mail address: gaetan.glauser@unine.ch (G. Glauser).

https://doi.org/10.1016/j.envpol.2019.02.004
0269-7491/© 2019 Elsevier Ltd. All rights reserved.

can already occur at concentrations in the range of 0.1–2 ppb (Moffat et al., 2015; Mitchell et al., 2017). As a comparison, the maximum residue limits (MRLs) set by the European Commission for human consumption range between 50 and 200 ppb in honey and other apiculture products.

Due to the increasing concerns about the effect of neonicotinoids on non-target organisms, a total and permanent ban vas voted in 2018 by the European Union regarding the outdoor use of the three most toxic neonicotinoids thiamethoxam, clothianidin and imidacloprid (EUR-Lex, 2018a,b,c). However, they will still be allowed indoor and there will be no restriction on the use of the other neonicotinoids. Canada is another example of a country implementing similar restrictions, but, in general, most governments have so far failed to take any action. Yet, according to many scientists, the situation is already critical and measures must be urgently taken to preserve biodiversity (Goulson, 2018). For example, a 2017 study revealed that more than 75% of the populations of flying insects have disappeared from Germany over the last three decades (Hallmann et al., 2017). Among the multiple factors which may be responsible for this erosion of insect biodiversity, agricultural intensification, in particular through the massive usage of pesticides, was recognized as a major possible cause. Moreover, we have recently shown that three quarters of the World's honeys are contaminated by at least one neonicotinoid, and that almost half contain levels which are potentially harmful to bees (i.e equal or superior to 0.1 ppb) (Mitchell et al., 2017). In that particular study, only the five most frequently used neonicotinoids were monitored: imidacloprid, clothianidin, thiamethoxam, acetamiprid, and thiacloprid (Fig. 1). However, at least four additional substances available on the global market may be considered as neonicotinoids or closely related pesticides: dinotefuran and nitenpyram (Fig. 1), which are employed both in agriculture and veterinary medicine (Elbert et al., 2008), and, importantly, two new types of insecticides, the sulfoximines (sulfoxaflor, Fig. 1) and the butenolides (flupyradifurone, Fig. 1), which are still subject to controversy whether they are neonicotinoids or not (Sparks and Nauen, 2015; Giorio et al., 2017). Sulfoximines and butenolides are structurally different and have been shown to potentially lack cross-resistance with conventional neonicotinoids (Longhurst et al., 2013; Nauen et al., 2015), but they share similar mechanisms of action as agonists of nicotinic acetylcholine receptors (Giorio et al., 2017). Moreover, sub-lethal effects on non-target insects have also been reported (Hesselbach and Scheiner, 2018; Siviter et al., 2018). Therefore, regardless of the classification being used, it seems reasonable to consider all of these molecules as potential threats for non-target insects and to concomitantly monitor their levels to

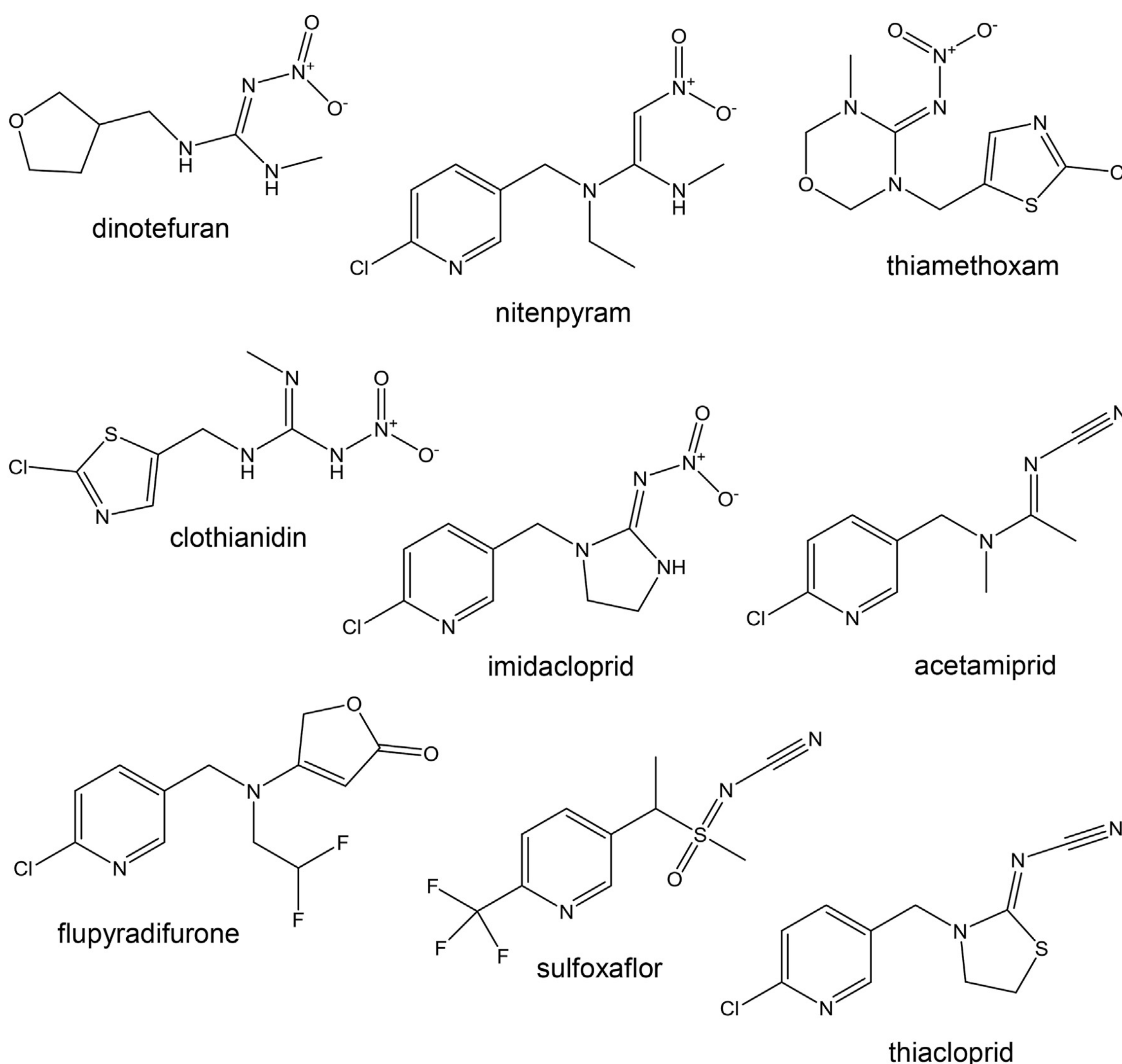


Fig. 1. Structures of the nine neonicotinoids and related pesticides analysed in this study.

establish a global picture of environment quality. In this regard, honey represents an ideal matrix: bees may travel up to 12.5 km from their hive, and thus the concentrations of insecticides in honey collected from a given hive are a measure of the contamination in the surrounding landscape (Mitchell et al., 2017). Moreover, honey samples are highly stable and can be easily collected and transported from various locations.

There have been several methods developed for the determination of neonicotinoids in honey (Kamel, 2010; Tanner and Czerwenka, 2011; Paradis et al., 2014; Mitchell et al., 2017). To our knowledge, however, no protocol able to simultaneously measure the nine molecules mentioned above at ultra-trace levels has been published. Here, we present a method able to accurately quantify these nine insecticides in honey at levels equal or inferior to 0.02 ppb, based on a QuEChERS (Quick, Easy, Cheap, Efficient, Rugged and Safe) procedure (Anastassiades et al., 2003) followed by UHPLC-MS/MS analysis. After development and optimisation, the method was validated for selectivity, limits of quantification (LOQ), response function, precision, accuracy, recovery and matrix effects. Finally, the method was applied to the analysis of 36 honey samples which had already been measured during our previous study (Mitchell et al., 2017), making possible a detailed examination of the long-term stability of neonicotinoids in honey for the first time.

2. Material and methods

2.1. Chemicals

For the extraction of samples, we used milli-Q water and acetonitrile (ACN) from VWR (Fontenay-sous-Bois, France). For UHPLC-MS/MS analyses, we employed water and acetonitrile of LC-MS grade from VWR (Fontenay-sous-Bois, France). Mobile phase additives, i.e. formic acid and ammonium formate, were of ULC-MS grade and purchased from Biosolve (Valkenswaard, The Netherlands). The salts used for QuEChERS were from Sigma-Aldrich (Buchs, Switzerland), the Isolute PSA bulk phase was from Biotage (Uppsala, Sweden), and the C18 (ZeoPrep 90) bulk phase was from ZeoChem (Uetikon, Switzerland). Thiamethoxam, clothianidin, imidacloprid, acetamiprid, thiacloprid, dinotefuran, nitenpyram, and flupyradifurone standards were obtained from Sigma-Aldrich. Sulfoxaflor was obtained from Clearsynth (Mumbai, India). Thiamethoxam-d3, clothianidin-d3, imidacloprid-d4, acetamiprid-d3 and thiacloprid-d4 were purchased at CDN Isotopes (Vaudreuil, Canada). Dinotefuran-d3 was purchased at EQ Laboratories GmbH (Augsburg, Germany). Nitenpyram-^{13}C-d3 was obtained from Alsachim (Illkirch-Graffenstaden, France).

2.2. Honey samples

The Botanical Garden of Neuchâtel houses a collection of 600 + honey samples from various locations of the World which is still growing. All samples are stored in the dark both at room temperature and −20 °C. In the present study, 36 samples which had been previously analysed and were shown to contain variable amounts of the five most frequent neonicotinoids (Mitchell et al., 2017) were selected and reanalysed. For samples stored at −20 °C, an aliquot was rapidly taken from the frozen pots without homogenisation to prevent thawing. For samples stored at room temperature, the entire pot was placed in a water bath at 40 °C and its content mixed with a spatula before sampling.

2.3. Sample preparation

The preparation of honey samples was based on QuEChERS and adapted from (Mitchell et al., 2017). A mass of 0.5 g of honey was weighed in a 15 mL polypropylene tube (Sartsedt, Nümbrecht, Germany). To the tube were added 9 mL of H_2O:ACN (50:50, v/v) and 20 μL of internal standard (IS) solution (125 ng/mL) containing the seven labelled neonicotinoids. The tube was placed in an ultrasonic bath for 10 min and then manually shaken until the solution was homogenous. The solution was transferred into a second 15 mL tube containing 2 g of $MgSO_4$, 0.5 g of NaCl, 0.5 g of sodium citrate dihydrate and 0.25 g of sodium citrate sesquihydrate. The first tube was washed with 1 mL of H_2O:ACN (50:50, v/v) and its content added to the second tube. The resulting mixture was vigorously hand-shaken for about 2 min, then centrifuged at 4000 *g* for 5 min. The epiphase was collected in a third 15 mL tube containing 0.15 g $MgSO_4$, 0.1 g PSA bulk phase and 0.1 g C18 bulk phase for solid phase dispersive extraction. Again, the tube was shaken for approximately 2 min and centrifuged at 4000 *g* for 5 min. The supernatant was recovered into a 13 × 100 mm glass tube and evaporated in a CentriVap centrifugal evaporator (Labconco, Kansas City, MO) at 35 °C. The dried residue was re-suspended in 0.5 mL of MeOH 25%, the tubes were vortexed, ultrasonicated for 2 min, and filtered through PTFE hydrophilic syringe filters (13 mm, 0.22 μm porosity, BGB Analytik, Böckten, Switzerland) into HPLC vials containing 250 μL conical inserts.

2.4. UHPLC-MS/MS analysis

The analysis of neonicotinoids was performed on a Waters system composed an Acquity UPLC system (Waters, Milford, MA) coupled to a TQ-S triple quadrupole (Waters), both controlled by Masslynx 4.1. Regarding the chromatographic part, a Cortecs UPLC C18 + column (100 mm × 2.1 mm i.d., 1.6 μm, Waters) was employed at a flow rate of 0.4 mL/min. The temperature of the column was kept at 25 °C. The analysis was performed in gradient mode using H_2O containing 0.05% formic acid and 5 mM ammonium formate as mobile phase A and acetonitrile containing only 0.05% formic acid as mobile phase B. The gradient parameters were as follows: 2–30.5% B in 7.5 min, 30.5–100% B in 0.5 min, held at 100% B for 2.0 min, reequilibration at 2% B for 4.0 min, for a total run time of 14 min. The volume of injection was 5 μL using a 10 μL loop in the Partial Loop with Needle Overfill (PLNO) mode. The flow was diverted from the MS from 0 to 2.4 min and from 8.45 to 14.0 min. Data was acquired in electrospray positive ionization with the capillary voltage set to +1.5 kV, the source temperature to 150 °C, the desolvation gas flow to 1000 L/h, the cone gas flow to 150 L/h, the nebuliser gas flow to 7 bars, and the desolvation gas temperature to 550 °C. The triple quadrupole was used in the multiple reaction monitoring (MRM) mode. One quantifier (Q) and one to two qualifier (q) transitions for each analyte were monitored, while only one transition per labelled internal standard was used, resulting in a total of 28 transitions. Optimized compound-dependent MS parameters are listed in Table 1.

2.5. Quantitation

The integration of chromatographic peaks was performed with the software Quanlynx (Waters). Calibration curves were built using concentrations at 2, 5, 20, 50, 500 pg/mL, 5 and 15 ng/mL in MeOH 25%. Each concentration solution contained all labelled standards at a constant concentration of 5 ng/mL. Analyte peaks were normalized to those of their corresponding labelled forms except for sulfoxaflor and flupyradifurone for which no isotopically labelled standards were available. Sulfoxaflor was then normalized to thiacloprid-d3 and flupyradifurone to acetamiprid-d3. Linear regressions weighted by 1/x were applied in all cases.

Table 1
Chromatographic and mass spectrometric parameters for the nine neonicotinoids and related molecules.

Analyte	RT (min)	CV (V)	Transition 1 (quantifier)	CE 1 (V)	Transition 2 (qualifier)	CE 2 (V)	Dwell time (ms)
Dinotefuran	2.85	30	203 → 157	5	203 → 113	10	67
Dinotefuran d-3	2.84	30	206 → 160	5	–	–	67
Nitenpyram	3.36	25	271 → 130	10	271 → 126[a]	27	67
Nitenpyram ^{13}C-d3	3.35	25	275 → 134	10	–	–	67
Thiamethoxam	4.46	25	292 → 211	12	292 → 132[a]	20	122
Thiamethoxam d-3	4.45	25	295 → 214	12	–	–	122
Clothianidin	5.22	30	250 → 169	12	250 → 132	12	80
Clothianidin d-3	5.20	30	253 → 172	13	–	–	80
Imidacloprid	5.54	35	256 → 209	15	256 → 175	20	80
Imidacloprid d-4	5.51	35	260 → 213	15	–	–	80
Acetamiprid	6.16	40	223 → 126	20	223 → 56	14	163
Acetamiprid d-3	6.13	40	226 → 126	20	–	–	163
Flupyradifurone	6.71	25	289 → 126	20	289 → 90	40	58
Sulfoxaflor	6.99	40	174 → 154	16	174 → 104[a]	25	58
Thiacloprid	7.31	40	253 → 126	20	253 → 90	34	58
Thiacloprid d-4	7.29	40	257 → 126	20	–	–	58

RT, retention time, CV, cone voltage, CE, collision energy.
[a] A second qualifier transition was used for thiamethoxam (292 → 181), nitenpyram (271 → 56) and sulfoxaflor (278 → 174).

2.6. Validation

An assay validation was performed according to SANTE/11813/2017 guidelines using a honey sample (#JBN313) which contained no quantifiable trace of neonicotinoids as blank matrix. Selectivity, response function and calibration range, lowest limit of quantitation (LLOQ), precision, accuracy, recovery, and matrix effects were determined. Selectivity was assessed by analysing the blank honey #JBN313 and the same honey spiked with a mixture of the nine neonicotinoids at 1 ppb. The response function of the calibration curve was evaluated by injecting 11 standard solutions ranging from 0.5 pg/mL to 50 ng/mL. Back-calculated concentrations were calculated and accepted if they were within ±20% of the true concentrations. The LLOQ was defined as the lowest concentration in spiked samples which could still be reliably quantified with acceptable accuracy (bias 75–125%) and precision (percentage of relative standard deviation (%RSD) ≤ 20%). Precision and accuracy were determined at 4 different concentrations using five replicates for each concentration (Table 2), and expressed as %RSD and percentage of deviation between actual and measured concentrations (bias), respectively. Extraction recovery was calculated as the ratio (in %) between honey samples (n = 5) spiked with equivalent amounts of neonicotinoids before and after extraction (corresponding to a final concentration of 0.5 ppb). Finally, matrix effects were calculated as the ratio between honey samples (n = 5) spiked after extraction and a stock solution of identical concentration (0.5 ppb).

2.7. Statistical analyses

Regression analyses were performed in GraphPad Prism4.

Table 2
Concentrations of the various analytes used in the validation assay.

Analyte	Conc. 1 (pg/g)	Conc. 2 (pg/g)	Conc. 3 (pg/g)	Conc. 4 (ng/g)
Dinotefuran	20	50	500	10
Nitenpyram	10	20	500	10
Thiamethoxam	5	10	500	10
Clothianidine	10	20	500	10
Imidacloprid	10	20	500	10
Acetamiprid	2	5	500	10
Flupyradifurone	5	10	500	10
Sulfoxaflor	5	10	500	10
Thiacloprid	2	5	500	10

3. Results and discussion

3.1. Sample preparation

The QuEChERS approach is the method of choice in pesticide analysis, including neonicotinoids. Several QuEChERS protocols exist, which slightly differ in the composition and proportion of salts employed. As the EN 15662 method has proved to provide the best recoveries for the most common neonicotinoids in honey (Paradis et al., 2014), it was selected and adapted in the present work. We used a mass of honey of only 0.5 g since we noticed that higher amounts could generate significant matrix effects during the analysis. Due to the small amount of honey, we reduced all solvent volumes and salt amounts so the entire procedure could be downscaled to 15 mL tubes. Average recoveries for the different pesticides ranged between 73% (SD 6%, n = 5) for nitenpyram and 95% (SD 4%, n = 5) for imidacloprid (see section 3.3 for specific recovery rates for each analyte). As a comparison, we also tested a liquid-liquid extraction (LLE) based on two extractions with ethylacetate, which provided excellent recoveries (86–95%) for most analytes except for dinotefuran (mean 58%, SD 5%, n = 3) and nitenpyram (mean 16%, SD 2%, n = 3). These low recoveries obtained by LLE are certainly due to the polar nature of these two pesticides. Altogether, these results show that the optimized QuEChERS method is appropriate for the extraction of the nine pesticides, including the most polar ones.

3.2. UHPLC-MS/MS conditions and matrix effects

A stepwise optimisation of the parameters of the MS source and collision cell was performed using flow injection analysis. In total, 28 MRM transitions were set and the chromatogram was divided in five acquisition segments to maximize dwell times (Table 1). While ions of the molecular species $[M+H]^+$ were selected as precursor ions for most molecules, sulfoxaflor exhibited strong in-source fragmentation with a prominent fragment at *m/z* 174 and almost no $[M+H]^+$ ion. Such phenomenon likely occurred in the funnel region (Stepwave™) of the instrument and could not be prevented by any means. The fragment at *m/z* 174 further fragmented into *m/z* 154 with excellent sensitivity and selectivity. A high-resolution mass spectrum obtained on a quadrupole time-of-flight system confirmed the cleavage of sulfoxaflor in α position of the sulfoximine group yielding *m/z* 174 and the subsequent loss of HF to yield *m/z* 154 (Fig. 2). Therefore, we selected the transition *m/z* 174 > 154

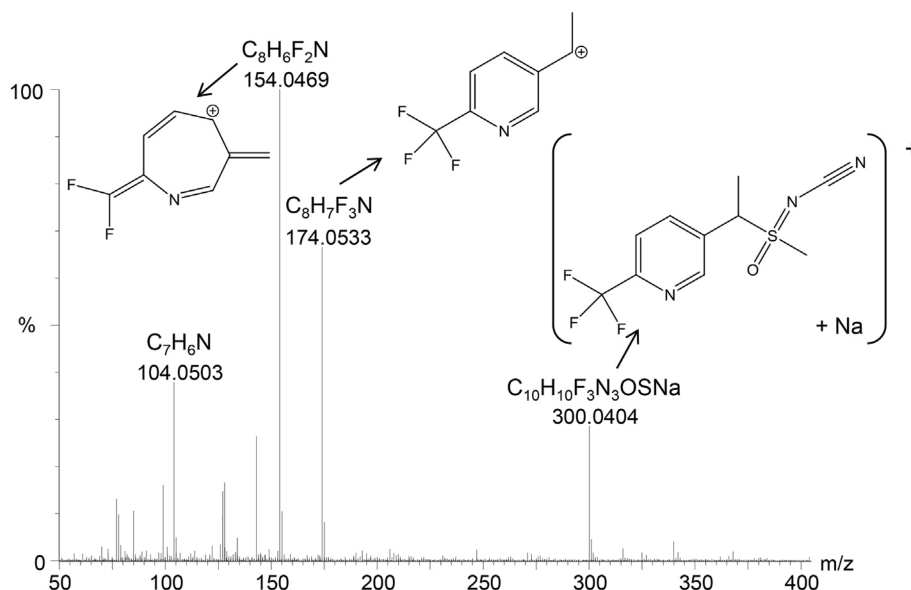


Fig. 2. High resolution mass spectrum of sulfoxaflor showing the absence of pseudo-molecular ion and the presence of two major fragments. Tentative structures for both fragments are presented.

for the detection of sulfoxaflor. It should be noted that, when UHPLC was coupled to mass spectrometry, sulfoxaflor eluted as two peaks separated by more than 0.2 min (Fig. 3). Indeed, this insecticide possesses two chiral centers and is commercialized as a mixture of isomers (Chen et al., 2014). Only the first peak was considered for quantification.

We then assessed the effect of adding ammonium formate to the mobile phase on the MS signals. Indeed, in our previous work on neonicotinoids, we found that addition of 5 mM ammonium formate to the aqueous acidic mobile phase provided a gain in signal-to-noise (s/n) ratio of 5–16-fold for the five most common molecules (Lachat and Glauser, 2018). Under the present conditions,

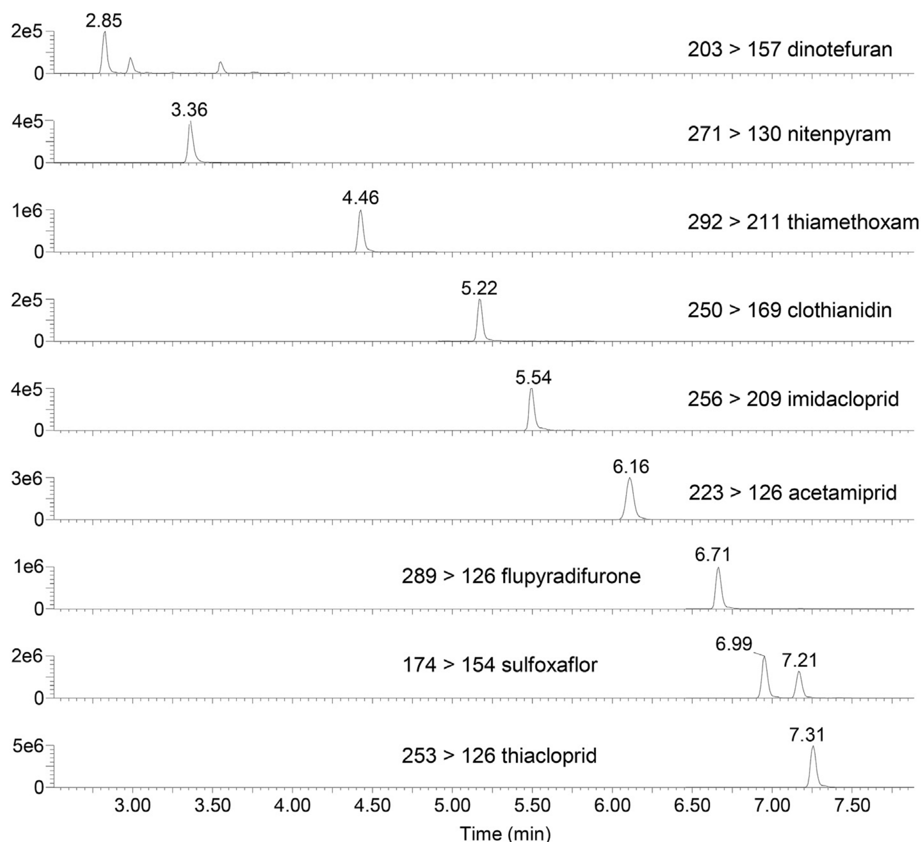


Fig. 3. Extracted ion chromatograms of a blank honey spiked with each analyte at a concentration of 1 ng/g.

this tendency was again confirmed with a 4–16-fold increase in s/n ratio for the five molecules and also for dinotefuran (9-fold increase in s/n ratio), nitenpyram (11-fold), sulfoxaflor (7-fold) and flupyradifurone (4-fold). Thus, the addition of ammonium formate to the mobile phase can be recommended for all neonicotinoids and related molecules, at least for this type of mass spectrometer.

In our previous studies, the separation of neonicotinoids was achieved either on a 50 mm Acquity UPLC BEH C18 column (Mitchell et al., 2017) or later on a 50 mm Cortecs UPLC C18 (Lachat and Glauser, 2018). Since honeys can be very variable in terms of textures and colours, and thereby contents, we spiked after extraction six different honeys of our collection which did not contain any trace of analytes to evaluate matrix effects over a relatively wide range of samples. With the 50 mm Cortecs C18 column and the chromatographic conditions previously applied (Lachat and Glauser, 2018), we sometimes observed strong matrix effects, in particular for the most polar neonicotinoids (Table 3). For instance, dinotefuran exhibited up to 74% signal reduction, nitenpyram up to 31%, thiamethoxam up to 39%, clothianidin up to 52%, and acetamiprid up to 53%. As an attempt to decrease matrix effects, we then selected the 100 mm Cortecs C18 + and extended the gradient time (Fig. 3). Under optimal conditions, the total run time was still very reasonable (14 min) and matrix effects were significantly reduced (Table 3), with a maximal signal decrease of 35% for dinotefuran, 21% for nitenpyram, 26% for thiamethoxam, 21% for clothianidin, and 17% for acetamiprid. Sulfoxaflor and flupyradifurone always displayed negligible or very minor matrix effects (<15%). Hence, we assumed that labelled internal standards would not be necessary for these two analytes. On the basis of the matrix effect and retention time closeness, we selected acetamiprid-d3 as internal standard for flupyradifurone and thiacloprid-d4 for sulfoxaflor.

3.3. Method validation

The method was validated using the blank honey sample #JBN313 which did not contain any detectable trace of neonicotinoids. Selectivity was shown by spiking this blank honey with each neonicotinoid at 1 ng/g, which resulted in no interfering peak for any of the analytes (Fig. 3). Selectivity was also confirmed by analysing the six honey samples used to determine matrix effects (see above) without spiking. The calibration ranges spanned over 4 to 5 orders of magnitude, with all back calculated concentrations within ± 15% and 10% at LLOQs and above, respectively (Table 4 and Supplementary Tables S1–S9). For all analytes but acetamiprid and thiacloprid, the highest calibration point was 50 ng/mL. In the case of acetamiprid and thiacloprid, the detector saturated at this concentration, and the highest calibration point was 10 ng/mL. Subsequent assays showed that the maximal concentrations which do not cause detector saturation are 25 and 15 ng/mL for acetamiprid and thiacloprid, respectively. Precision and accuracy were determined at four different concentrations and fell within the acceptable limits at all concentrations (Table 4) except for two cases: accuracy for flupyradifurone at the lowest concentration of 5 pg/g was 132%, and therefore the method LLOQ for this analyte was set to 10 pg/g. Furthermore, the relative standard deviation for thiacloprid at 5 pg/g was 22%. Yet, accuracy at this concentration was acceptable (111%), and the precision and accuracy at the lowest concentration of 2 pg/g were still within the limits (Table 4). Despite this out-of-range precision value at 5 pg/g, the method LLOQ for thiacloprid was thus defined as 2 pg/g. Overall, method LLOQs ranged from 2 pg/g for acetamiprid and thiacloprid to 20 pg/g for dinotefuran (Table 4). These LLOQs are 1–3 orders of magnitude lower than those previously reported by other authors (Kamel, 2010; Tanner and Czerwenka, 2011; Jovanov et al., 2013; Paradis et al., 2014) and even better than those from our previous own study (Mitchell et al., 2017) even though the amount of starting material has been reduced by 5-fold. For all analytes, LLOQS are below the concentration of 100 pg/g expected to be potentially harmful to bees. Extraction recoveries ranged between 73 and 95% for the different analytes and, thanks to the selective QuEChERS procedure and the optimisation of the chromatographic separation, matrix effects were insignificant (Table 4). Altogether, the validation demonstrates that

Table 3
Matrix effects in % obtained on the 100 mm C18 + Cortecs column (bold) and on the 50 mm C18 Cortecs column (in brackets). Samples were spiked after extraction with a mixture of pesticides at 0.5 ppb and the obtained signals compared with those of the pure solution.

Analyte	Sample #JBN308	Sample #JBN334	Sample #JBN335	Sample #JBN446	Sample #JBN296	Sample #JBN313
Dinotefuran	**83** (67)	**65** (26)	**68** (56)	**76** (66)	**87** (88)	**78** (76)
Nitenpyram	**96** (84)	**79** (69)	**102** (87)	**94** (83)	**117** (97)	**107** (93)
Thiamethoxam	**105** (75)	**81** (61)	**74** (82)	**102** (85)	**101** (93)	**92** (84)
Clothianidine	**129** (94)	**79** (48)	**86** (63)	**107** (77)	**98** (92)	**88** (81)
Imidacloprid	**113** (113)	**104** (93)	**106** (119)	**139** (114)	**79** (101)	**87** (117)
Acetamiprid	**93** (84)	**88** (47)	**90** (90)	**90** (73)	**91** (104)	**83** (89)
Flupyradifurone	**97** (82)	**112** (94)	**102** (100)	**99** (81)	**99** (92)	**89** (110)
Sulfoxaflor	**91** (88)	**89** (83)	**95** (95)	**86** (84)	**97** (98)	**85** (88)
Thiacloprid	**100** (98)	**94** (82)	**97** (104)	**92** (97)	**99** (98)	**85** (87)

Table 4
Validation parameters for the quantitation of neonicotinoids and related molecules in honey.

Analyte	Calibration range	LLOQ (pg/g)	Precision (%RSD, n = 5)				Accuracy (%, n = 5)				ER	ME
	(pg/mL)		Conc. 1	Conc. 2	Conc. 3	Conc. 4	Conc. 1	Conc. 2	Conc. 3	Conc. 4	(%)	(%)
Dinotefuran	10–50000	20	5	13	2	5	93	111	106	111	84	75
Nitenpyram	5–50000	10	7	9	4	4	101	109	101	108	73	86
Thiamethoxam	1–50000	5	8	7	2	4	113	103	108	106	88	84
Clothianidin	5–50000	10	9	7	4	4	112	105	98	106	90	81
Imidacloprid	5–50000	10	6	11	4	2	112	100	115	111	95	96
Acetamiprid	0.5–25000	2	9	7	1	5	116	109	108	106	90	85
Flupyradifurone	5–50000	10	11	4	3	4	132	116	115	105	91	88
Sulfoxaflor	1–50000	5	11	4	5	3	101	99	105	106	91	93
Thiacloprid	0.5–15000	2	7	22	1	4	112	111	106	112	91	93

LLOQ, lowest limit of quantification, %RSD, relative standard deviation, ER, extraction recovery, ME, matrix effects.

the method is able to reliably quantify all analytes both at very low and high concentrations, and may thus be applied to the analysis of honey samples from various origins.

3.4. Stability study

The developed method was applied to the analysis of 36 honeys from different regions of the World which had already been analysed for imidacloprid, thiamethoxam, clothianidin, acetamiprid and thiacloprid over the period 2015–2017 (Mitchell et al., 2017). These samples were selected from a larger collection as they presented variable levels of the different neonicotinoids. Since honeys had been measured according to a fully validated protocol and further stored both at room temperature and −20 °C, this

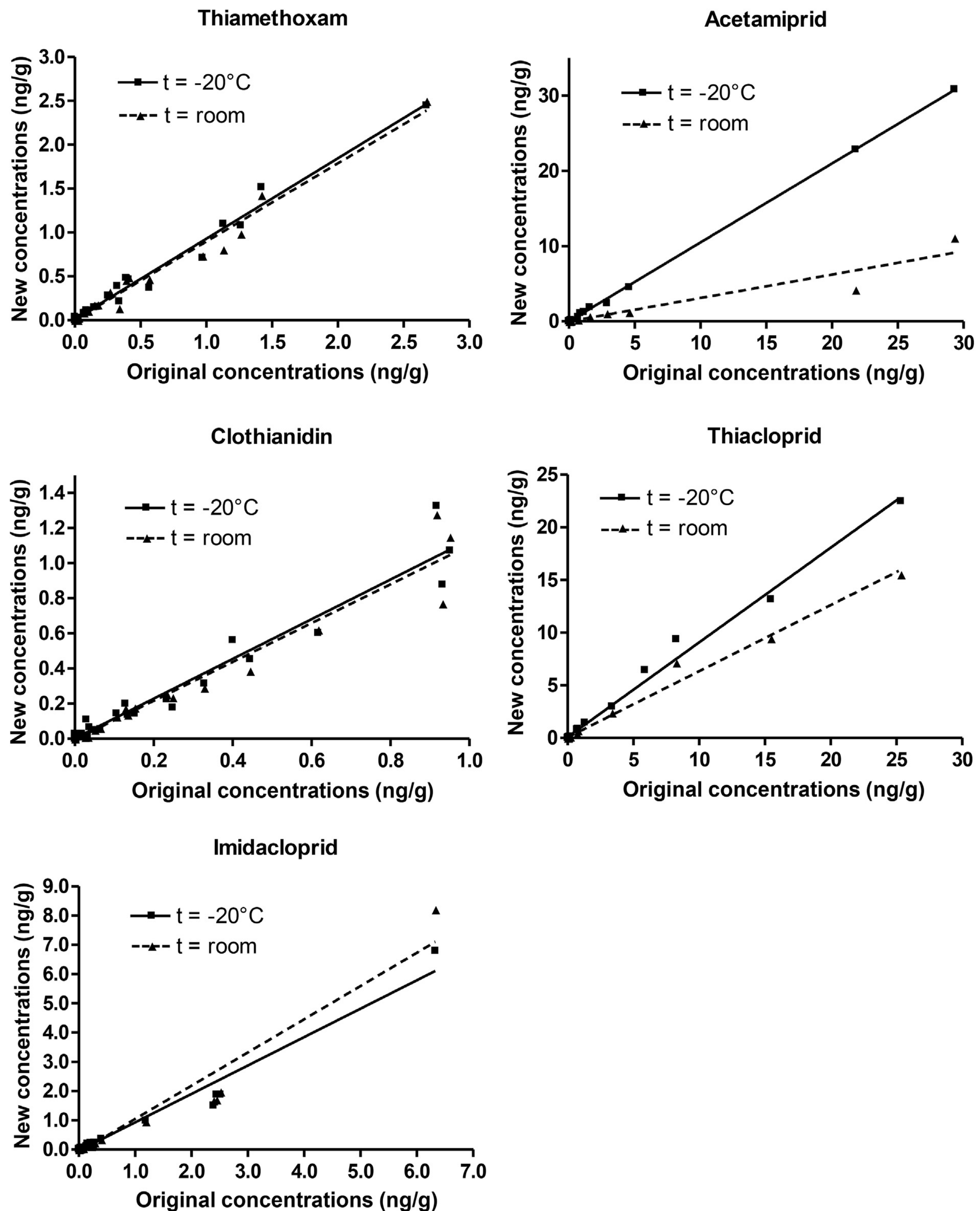


Fig. 4. Regression lines obtained by plotting concentrations in samples stored at −20 °C (solid line) or room temperature (dashed line) *versus* original concentrations obtained from our previous study (Mitchell et al., 2017).

represented a unique opportunity to determine the long-term (up to 40 months) stability of neonicotinoids in a relatively stable matrix under controlled storage conditions. The concentrations obtained for samples stored at room temperature and −20 °C were compared with the original concentrations using linear regressions. Three parameters were evaluated, namely the slope and intercept of the regression line, as well as the coefficient of determination R^2. One sample (#JBN210) exhibited concentrations ca. twice higher for all analytes in the previous analysis compared to the two new analyses and was thus excluded from the dataset. As can be seen in Fig. 4 and Table 5, all regressions lines obtained for samples stored at −20 °C had slopes close to 1, with intercept close to 0 and R^2 close to 1. This indicates that results were highly correlated and that the five compounds are stable under frozen conditions. By contrast, storage at room temperature resulted in slopes inferior to 1 for acetamiprid (slope = 0.31) and thiacloprid (slope = 0.62), while the other three pesticides had slopes close to 1. In other words, imidacloprid, thiamethoxam and clothianidin are stable in honey at room temperature for several years, but acetamiprid and thiacloprid partially degrade under these conditions. Such degradation was certainly due to the acidity of honey and the fact that, contrary to the three former molecules, the two latter are unstable in acidic media (Guzsvány et al., 2006). That molecules were stable in the freezer suggests that degradation was prevented at low temperature. We did not find any relationship between the age of honey and the level of degradation. The complete result table for the stability study can be consulted in Supplementary Table S10. The fact that acetamiprid and thiacloprid both degrade at room temperature suggests that the measurement of concentrations in our previous study for these two neonicotinoids (Mitchell et al., 2017) was potentially substantially lower that the concentrations existing in the beehive at harvest. To avoid such bias is it therefore preferable to store samples at −20 °C if possible until they can be analysed.

3.5. Concentrations of the other neonicotinoids and related molecules

We also verified the levels of the four insecticides which could not be monitored with our previous method, namely dinotefuran, nitenpyram, sulfoxaflor, and flupyradifurone. Among the 36 investigated honeys, 10 (i.e. 28%) contained at least one of these compounds. This is much less than the rate of contamination (75%) found for the five most common neonicotinoids in our previous study (Mitchell et al., 2017). Specifically, 5 samples contained dinotefuran, 6 flupyradifurone, 7 sulfoxaflor, and none was contaminated with nitenpyram (Supplementary Table S10). Concentrations were very low, ranging between 5 and 281 pg/g, except for two samples. The first one originated from Japan and contained 2.4 ng/g of dinotefuran. Dinotefuran was registered in Japan in 2002,

Table 5
Parameters of the linear regressions obtained for the stability study.

Analyte	Temperature	Slope	Intercept	R^2
Thiamethoxam	−20 °C	0.916	0.013	0.983
	Room temp.	0.891	0.005	0.979
Clothianidin	−20 °C	1.131	0.002	0.953
	Room temp.	1.106	−0.005	0.949
Imidacloprid	−20 °C	0.972	−0.044	0.968
	Room temp.	1.136	−0.093	0.942
Acetamiprid	−20 °C	1.049	−0.014	0.999
	Room temp.	0.311	−0.018	0.919
Thiacloprid	−20 °C	0.900	0.083	0.992
	Room temp.	0.628	0.058	0.988

and it is thus not surprising to find this neonicotinoid in honeys from this country. Corroborating this observation, two other Japanese honeys also contained dinotefuran (Supplementary Table S10). The second sample was from Florida and contained 5.0 ng/g sulfoxaflor and 45.9 ng/g flupyradifurone, in addition to 2.5 ng/g of thiamethoxam, 1.3 ng/g of clothianidin, and 6.8 ng/g of imidacloprid. The total neonicotinoid content in this sample was thus 61.4 ng/g, which is more than 30 times superior to the average content found in our previous global survey (Mitchell et al., 2017). It was collected in April 2016, while sulfoxaflor and flupyradifurone have been brought on the U.S. market in 2013 and 2015, respectively. This suggests that certain farmers had already started to massively use these novel insecticides shortly after their authorisation on the U.S territory. With the recent ban recently implemented in the European Union, it will be interesting to determine if a shift from conventional neonicotinoids to sulfoximines and butenolides can be detected in the upcoming years in Europe. The fact that high concentrations of some molecules not included in our previous study (Mitchell et al., 2017) were recorded in regions where the total concentration of the other five molecules was already high (e.g. Japan and U.S.) is worrying and indicates potentially high usage of pesticides in agricultural production.

4. Conclusion

The developed method is able to determine the presence in honey of all parent neonicotinoids with unprecedented level of sensitivity and excellent precision and accuracy. Such sensitivity is essential for ecological studies on non-target insects as sub-lethal effects may be observed at concentrations that are much lower than those assumed to be safe for human consumption. A future objective will be to extend the method to the analysis of relevant neonicotinoid metabolites in order to get a broader view of honey contamination by these pesticides. This will be particularly significant for unstable molecules such as acetamiprid and thiacloprid.

Author contributions

B.M., A.A, E.M and G.G conceived the study. S.K. and G.G performed the experiements, analysed and interpreted data. G.G wrote the first draft and all authors read and approved the manuscript.

Conflict of interest disclosure

The authors declare no conflict of interest.

Acknowledgements

We thank all the persons who provided honey samples. We are also grateful to José Richard, Laurence Lachat, Claudia di Césaré and Dragan Dalaveraj for technical assistance and to Stephan von Reuss and Armelle Vallat for helpful discussion. This work was supported by the University of Neuchatel.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2019.02.004.

References

Anastassiades, M., Lehotay, S.J., Stajnbaher, D., Schenck, F.J., 2003. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. J. AOAC Int. 86 (2), 412–431.
Bonmatin, J.-M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.,

Liess, M., Long, E., Marzaro, M., Mitchell, E.A.D., Noome, D.A., Simon-Delso, N., Tapparo, A., 2015. Environmental fate and exposure; neonicotinoids and fipronil. Environ. Sci. Pollut. Res. 22 (1), 35–67.
Chen, Z., Dong, F., Xu, J., Liu, X., Cheng, Y., Liu, N., Tao, Y., Zheng, Y., 2014. Stereoselective determination of a novel chiral insecticide, sulfoxaflor, in Brown Rice, Cucumber and Apple by normal-phase high-performance liquid chromatography. Chirality 26 (2), 114–120.
Elbert, A., Haas, M., Springer, B., Thielert, W., Nauen, R., 2008. Applied aspects of neonicotinoid uses in crop protection. Pest Manag. Sci. 64 (11), 1099–1105.
EUR-Lex, 2018a. Commission Implementing Regulation (EU) 2018/783 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance imidacloprid. Off. J. Eur. Union L132, 31–34.
EUR-Lex, 2018b. Commission Implementing Regulation (EU) 2018/784 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance clothianidin. Off. J. Eur. Union L132, 35–39.
EUR-Lex, 2018c. Commission Implementing Regulation (EU) 2018/785 of 29 May 2018 amending Implementing Regulation (EU) No 540/2011 as regards the conditions of approval of the active substance thiamethoxam. Off. J. Eur. Union. L132, 40–44.
Giorio, C., Safer, A., Sánchez-Bayo, F., Tapparo, A., Lentola, A., Girolami, V., van Lexmond, M.B., Bonmatin, J.-M., 2017. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 1: new molecules, metabolism, fate, and transport. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-017-0394-3.
Goulson, D., 2013. An overview of the environmental risks posed by neonicotinoid insecticides. J. Appl. Ecol. 50 (4), 977–987.
Goulson, D., 2018. Call to restrict neonicotinoids. Science 360 (6392), 973-973.
Guzsvány, V., Csanádi, J., Gaal, F., 2006. NMR study of the influence of pH on the persistence of some neonicotinoids in water. Acta Chim. Slov. 53, 52–57.
Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D., de Kroon, H., 2017. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. PLoS One 12 (10), e0185809.
Hesselbach, H., Scheiner, R., 2018. Effects of the novel pesticide flupyradifurone (Sivanto) on honeybee taste and cognition. Sci. Rep. 8 (1), 4954.
Jeschke, P., Nauen, R., Schindler, M., Elbert, A., 2011. Overview of the status and global strategy for neonicotinoids. J. Agric. Food Chem. 59 (7), 2897–2908.
Jovanov, P., Guzsvány, V., Franko, M., Lazić, S., Sakač, M., Šarić, B., Banjac, V., 2013. Multi-residue method for determination of selected neonicotinoid insecticides in honey using optimized dispersive liquid–liquid microextraction combined with liquid chromatography-tandem mass spectrometry. Talanta 111, 125–133.
Kamel, A., 2010. Refined methodology for the determination of neonicotinoid pesticides and their metabolites in honey bees and bee products by liquid chromatography–tandem mass spectrometry (LC-MS/MS). J. Agric. Food Chem. 58 (10), 5926–5931.
Lachat, L., Glauser, G., 2018. Development and validation of an ultra-sensitive UHPLC–MS/MS method for neonicotinoid analysis in milk. J. Agric. Food Chem. 66 (32), 8639–8646.
Longhurst, C., Babcock, J.M., Denholm, I., Gorman, K., Thomas, J.D., Sparks, T.C., 2013. Cross-resistance relationships of the sulfoximine insecticide sulfoxaflor with neonicotinoids and other insecticides in the whiteflies Bemisia tabaci and Trialeurodes vaporariorum. Pest Manag. Sci. 69 (7), 809–813.
Mitchell, E.A.D., Mulhauser, B., Mulot, M., Mutabazi, A., Glauser, G., Aebi, A., 2017. A worldwide survey of neonicotinoids in honey. Science 358 (6359), 109–111.
Moffat, C., Pacheco, J.G., Sharp, S., Samson, A.J., Bollan, K.A., Huang, J., Buckland, S.T., Connolly, C.N., 2015. Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (Bombus terrestris). FASEB J. 29 (5), 2112–2119.
Nauen, R., Jeschke, P., Velten, R., Beck, M.E., Ebbinghaus-Kintscher, U., Thielert, W., Wölfel, K., Haas, M., Kunz, K., Raupach, G., 2015. Flupyradifurone: a brief profile of a new butenolide insecticide. Pest Manag. Sci. 71 (6), 850–862.
Paradis, D., Bérail, G., Bonmatin, J.-M., Belzunces, L.P., 2014. Sensitive analytical methods for 22 relevant insecticides of 3 chemical families in honey by GC-MS/MS and LC-MS/MS. Anal. Bioanal. Chem. 406 (2), 621–633.
Pisa, L., Goulson, D., Yang, E.-C., Gibbons, D., Sánchez-Bayo, F., Mitchell, E., Aebi, A., van der Sluijs, J., MacQuarrie, C.J.K., Giorio, C., Long, E.Y., McField, M., Bijleveld van Lexmond, M., Bonmatin, J.-M., 2017. An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 2: impacts on organisms and ecosystems. Environ. Sci. Pollut. Res. https://doi.org/10.1007/s11356-017-0341-3.
Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K., Desneux, N., 2016. Are bee diseases linked to pesticides? — a brief review. Environ. Int. 89–90, 7–11.
Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E.A.D., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van der Sluijs, J.P., Whitehorn, P.R., Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environ. Sci. Pollut. Res. 22 (1), 5–34.
Siviter, H., Brown, M.J.F., Leadbeater, E., 2018. Sulfoxaflor exposure reduces bumblebee reproductive success. Nature 561 (7721), 109–112.
Sparks, T.C., Nauen, R., 2015. IRAC: mode of action classification and insecticide resistance management. Pestic. Biochem. Physiol. 121, 122–128.
Tanner, G., Czerwenka, C., 2011. LC-MS/MS analysis of neonicotinoid insecticides in honey: methodology and residue findings in Austrian honeys. J. Agric. Food Chem. 59 (23), 12271–12277.