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LC-MS/MS Analysis of Neonicotinoid Insecticides in Honey: Methodology and Residue Findings in Austrian Honeys

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ABSTRACT: An analytical method for the simultaneous determination of residues of eight neonicotinoid insecticides and two metabolites in honey using LC-MS/MS was developed and validated. Two approaches of sample preparation were investigated, with the final method involving acetonitrile extraction and subsequent cleanup by dispersive solid-phase extraction (QuEChERS type). Validation was based on quintuplicate analysis at three fortification levels and showed satisfactory recoveries (60−114%) and high precision (RSDs between 2.7 and 12.8%). Low limits of detection and quantification could be achieved for all analytes ranging from 0.6 to 5 μg/kg and from 2 to 10 μg/kg, respectively. Investigations of Austrian honey samples revealed the presence of acetamiprid, thiacloprid, and thiamethoxam residues in honey; however, no sample exceeded the maximum residue limits. On average, flower honey samples contained neonicotinoid residues in higher quantities compared to forest honey samples.

KEYWORDS: neonicotinoid insecticides, pesticide residues, honey, QuEChERS, LC-MS/MS

INTRODUCTION

The relatively new group of neonicotinoids constitutes a class of highly potent insecticides, which were developed in a series of syntheses from nitro-substituted ketene aminales.[1] The so-called first-generation neonicotinoids (acetamiprid, imidacloprid, nitenpyram, and thiacloprid) are characterized by a 6-chloro-3-pyridyle heterocycle, the second-generation compounds (clothianidin and thiamethoxam) contain a 2-chloro-5-thiazolyl moiety, whereas dinotefuran belongs to the third generation and features a 3-tetrahydrofuranyl group (Figure 1).[1] Additionally, flonicamid, which is characterized by a 4-trifluoromethyl-3-pyridyl group, is frequently assigned to the neonicotinoid group. New neonicotinoids are continuing to be developed to date.[2] Neonicotinoids act in a very specific way as agonists on the postsynaptic nicotinic acetylcholine receptor of the insect's central nervous system, causing a blockage of signal transmission. Distinct advantages of neonicotinoids are their high efficacy, selectivity, and plant systemicity as well as long-lasting effect and versatile application.[3] A further crucial factor for the success of neonicotinoids is the absence of a cross-resistance to longer-established insecticide classes such as carbamates, organophosphates, or synthetic pyrethroids, against which many pests have developed resistances over the years.[4,5] The versatile application of neonicotinoid insecticides covers many crops ranging from cereals and vegetables to various fruit cultures.

Upon the use of neonicotinoids as a measure of pest management, beneficial insects such as honeybees may also be affected. Depending on the application form of neonicotinoid insecticides, different routes of exposure of honeybees to these pesticides can be envisaged. The application of neonicotinoids as chemical sprays can contaminate the blossoms of plants on and beside agricultural fields as well as foraging honeybees during their flight. The same ways of exposure can also occur upon abrasion and environmental drift of neonicotinoids contained in seed dressings during the sowing process. Additionally, neonicotinoids applied in seed dressings are distributed in the plants, and honeybees might come into contact with them through their presence in pollen or nectar. When honeybees come into contact with neonicotinoids, the insecticides may be taken along into the beehive, and residues may finally be found in bee products such as honey. For different neonicotinoid residues maximum residue limits in honey have been set by the European Union (EU) ranging from 10 to 200 μg/kg (Table 1). The residue definitions of acetamiprid, flonicamid, and thiamethoxam also include one metabolite each (Table 1).

Due to the widespread application of neonicotinoid insecticides, appropriate analytical methods for the detection and quantification of their residues in honey are required. In recent years several publications have reported analytical methods for the analysis of pesticide residues in honey. A review of chromatographic methods[6] provided an overview of the approaches employed for the extraction of pesticide residues from honey as well as the chromatographic methods used to measure them. In terms of neonicotinoids, most of the reported multiresidue methods included one or more substances from this group of insecticides. With regard to methods focusing on the analysis of residues of the neonicotinoid group, papers have been published dealing with fruits and vegetables[7−10] as well as foodstuffs of animal origin.[9] Only a small number of publications have specifically targeted the analysis of neonicotinoid residues in honey.[11−13] Whereas the methods by Schöning and Schmuck[11] and Kamel[13] focused only on imidacloprid and its metabolites, Fidente et al.[12] included acetamiprid, imidacloprid, thiacloprid, and thiamethoxam in their method. In terms of sample preparation, different approaches were employed in these studies. Whereas Kamel[13] utilized a modified QuEChERS method[14] supplemented by subsequent solid phase extraction using a C_{18}

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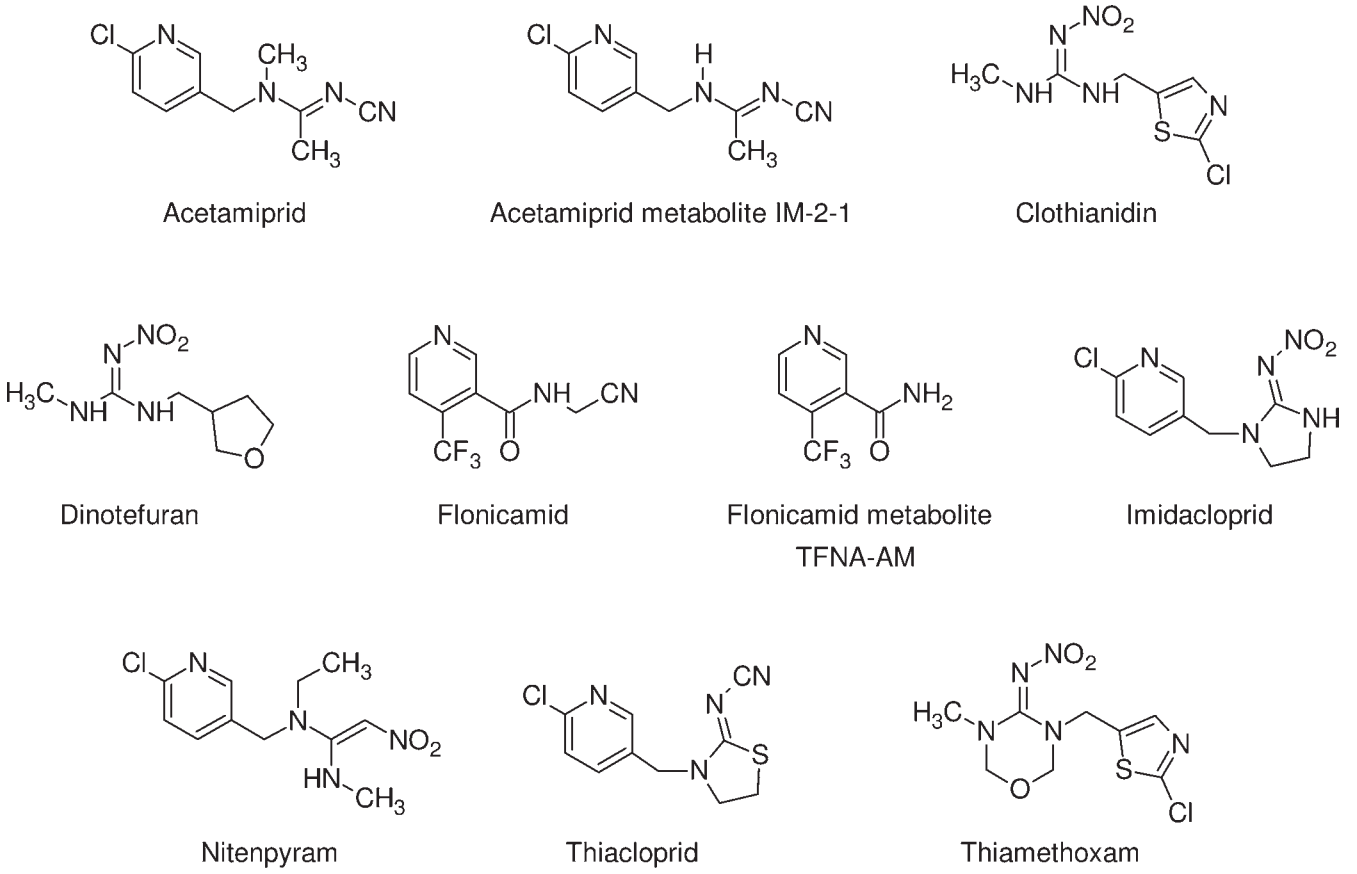

Figure 1. Chemical structures of investigated neonicotinoid insecticides and metabolites.

Table 1. Current Maximum Residue Limits (MRLs) and Residue Definitions for Neonicotinoid Insecticides in the European Union (as of January 10, 2011) [18]

substance (residue definition)	MRL in honey (μg/kg)
acetamiprid (sum of acetamiprid + IM 2-1)	50
clothianidin	10
dinotefuran	10[a]
flonicamid (sum of flonicamid + TFNA-AM)	50
imidacloprid	50
nitenpyram	10[a]
thiacloprid	200
thiamethoxam (sum of thiamethoxam + clothianidin)	10

[a] General MRL of 10 μg/kg due to the absence of a specific MRL for the substance in honey.

cartridge for optimum cleanup, the other methods used liquid−liquid extraction with dichloromethane [12] or cyclohexane/ethyl acetate,[11] respectively. Fidente et al.[12] used LC-MS as measurement technique, whereas in the other methods[11,13] LC-MS/MS was employed, which resulted in considerably lower limits of quantification compared to LC-MS.

To our knowledge no analytical method for the simultaneous determination of the entire group of neonicotinoid insecticides and their metabolites according to the EU residue definitions in honey has been published so far. In the present study we report the development and validation of a method tackling this analytical question and its application toward residue analysis of various honey samples.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents. Acetamiprid and its metabolite *N*-desmethylacetamiprid (IM 2-1) were purchased from Nippon Soda (Tokyo, Japan). Clothianidin, dinotefuran, flonicamid, imidacloprid, nitenpyram, and thiacloprid were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Thiamethoxam was from Riedel de Haën (Seelze, Germany), whereas the flonicamid metabolite 4-trifluoromethylnicotinamide (TFNA-AM) was from Fluorochem (Hadfield, U.K.). A stock solution of clothianidin-d_3 in acetonitrile (0.312 g/L) was provided by Bayer CropScience. Acetonitrile, sodium chloride, disodium hydrogen citrate sesquihydrate, trisodium citrate dihydrate, and Celite were purchased from VWR (Vienna, Austria), whereas methanol, cyclohexane, and ethyl acetate were obtained from LGC Standards (Wesel, Germany). Formic acid was purchased from Riedel de Haën, and anhydrous magnesium sulfate and primary−secondary amine (PSA) 40 μm were from Sigma-Aldrich (Steinheim, Germany). Deionized water was prepared with an in-house water purification system from Millipore (Billerica, MA).

Standards. Individual standard stock solutions of 1 g/L were prepared by dissolving 5−10 mg of the respective analyte (weighed with an accuracy of 0.01 mg) in the appropriate amount of acetonitrile. Stock solutions were stored at 4 °C and were stable for at least 1 year. The standard stock solutions were mixed and diluted with acetonitrile to obtain analyte mixture working solutions of all investigated analytes at levels of 10, 1, and 0.1 mg/L. Internal standard solutions of clothianidin-d_3 in acetonitrile were prepared at 10 and 1 mg/L by dilution of the stock solution.

Solvent standards in methanol with concentrations of 2, 5, 10, 25, 50, and 100 μg/L and the internal standard at 50 μg/L were prepared using the 1 or 0.1 mg/L working standard mixture, respectively, and the 1 mg/L internal standard solution.

Matrix-matched standards were prepared by using mountain flower honey from a local store as blank matrix. Blank honey was worked up according to the QuEChERS type sample preparation method (vide infra) without adding the internal standard solution at the beginning. Prior to the last solvent evaporation step, appropriate amounts of internal standard and analyte mixture working solutions were added for each standard level. Matrix-matched standards were prepared at levels of 2, 5, 10, 25, 50, and 100 μg/L with the internal standard at 50 μg/L.

Spiked Honey Samples. Mountain flower honey was used as blank matrix and spiked at three levels of 10, 50, and 100 μg/kg by adding the appropriate amount of the 10 mg/L analyte mixture working solution. The honey was then stirred for 30 min at 45 °C.

Honey Samples. All investigated honey samples originated from Austria and were produced in 2009. They were either commercial samples collected within the Austrian residue control program or originated from individual beehives, being supplied by the respective beekeeper via the Institute of Apiculture of the Austrian Agency for Health and Food Safety.

Sample Preparation. *ChemElut Type Method.* To 1 g of honey were added 10 mL of water and 50 μL of 1 mg/L internal standard solution, and the sample was placed in an ultrasonic bath for 2 min. After the addition of 20 mL of methanol, the sample was homogenized for 1 min with an Ultra-Turrax (IKA Labortechnik, Staufen, Germany). The sample was then filtered through a paper filter with 2.5 g of Celite as filtering aid. The filter was washed with 20 mL of methanol/water 75:25 (v/v). The filtrate was concentrated to the aqueous remainder on a rotary evaporator at 50 °C. The aqueous remainder was transferred onto a ChemElut 1020 column. After 15 min, elution was carried out with 80 mL of cyclohexane/ethyl acetate 50:50 (v/v). The eluate was evaporated to dryness on a rotary evaporator at 50 °C. The residue was redissolved in 2 mL of methanol. This solution was evaporated to dryness using a stream of nitrogen at 30 °C. Finally, the residue was redissolved in 1 mL of methanol/water 20:80 (v/v) and subjected to the LC-MS/MS analysis.

QuEChERS Type Method. Five grams of honey, 25 μL of 10 mg/L internal standard solution, 10 mL of water, and 10 mL of acetonitrile were mixed in a 50 mL centrifuge tube, which was then vigorously shaken by hand until a homogeneous solution was obtained. A mixture of 4 g of anhydrous magnesium sulfate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate was added to the tube. The tube was shaken vigorously by hand for 1 min and centrifuged for 5 min at 3000*g* and 10 °C. An aliquot of 6 mL of the acetonitrile phase was transferred into a Pyrex tube containing 900 mg of anhydrous magnesium sulfate and 150 mg of PSA. The tube was vigorously shaken by hand for 30 s and centrifuged for 5 min at 3000*g* and 10 °C. Two milliliters of the supernatant was evaporated to dryness using a stream of nitrogen at 30 °C. The residue was redissolved in 1 mL of methanol/water 20:80 (v/v) and subjected to LC-MS/MS analysis.

Analysis by LC-MS/MS. LC-MS/MS was performed using an Agilent 1100 HPLC (Agilent Technologies, Waldbronn, Germany) coupled to a PE SCIEX API 2000 triple-quadrupole mass spectrometer (MDS Sciex, Concord, Canada) equipped with an electrospray ion source. The analytes were separated on a Synergi Fusion RP column (50 × 2 mm, 4 μm) at 20 °C. The following gradient program using water with 5 mmol/L ammonium formate as mobile phase A and methanol with 5 mmol/L ammonium formate as mobile phase B was employed: 0 min, 10% B; 7 min, 38% B; 12 min, 90% B; followed by a 5 min washing step at 100% B. The flow rate was 200 μL/min and the injection volume 25 μL. The mass spectrometer was operated in positive ionization mode, and data were acquired in the selected reaction monitoring (SRM) mode with two transitions per compound. The source temperature was set at 400 °C, and the employed gas flows were as follows: nebulizer gas, 30 psi; heater gas, 70 psi; curtain gas, 30 psi; and collision gas, 5 psi. The transitions and potentials for each analyte are shown in Table 2. The LC-MS/MS system was controlled by Analyst 1.5 software. The transition with the highest intensity was used as quantifier and the second transition as qualifier. Quantification was based on 6-point calibrations (2−100 μg/L) using solvent standards for the ChemElut type method and solvent or matrix-matched standards for the QuEChERS type method employing clothianidin-d_3 as internal standard for all analytes.

Table 2. MS/MS Parameters for Both SRM Transitions of All Analytes

analyte	Q1[a]	Q3[b]	DP[c]	CE[d]
acetamiprid	223	126	36	27
	223	90	34	45
acetamiprid metabolite IM 2-1	209	126	26	23
	209	90	26	43
clothianidin	250	132	31	19
	250	169	31	19
clothianidin-d_3 (internal standard)	253	132	31	19
	253	172	31	19
dinotefuran	203	129	16	17
	203	113	16	15
flonicamid	230	203	31	21
	230	148	31	39
flonicamid metabolite TFNA-AM	191	148	26	31
	191	98	26	43
imidacloprid	256	209	51	21
	256	175	49	25
nitenpyram	271	126	26	45
	271	225	26	17
thiacloprid	253	126	81	29
	253	186	79	19
thiamethoxam	292	211	21	17
	292	181	21	31

[a] m/z of precursor ion. [b] m/z of product ion. [c] Declustering potential. [d] Collision energy.

RESULTS AND DISCUSSION

Method Development. With regard to analyte selection, all neonicotinoid insecticides currently available on the market as well as their metabolites according to the EU residue definitions for honey were included in the method (Figure 1). Thus, the investigated group of analytes consisted of eight neonicotinoid insecticides (acetamiprid, clothianidin, dinotefuran, flonicamid, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam) and

two metabolites (acetamiprid metabolite IM 2-1 and flonicamid metabolite TFNA-AM). In this context it is noted that clothianidin is at the same time a neonicotinoid insecticide and a metabolite of thiamethoxam. An isotopically labeled variant of clothianidin, clothianidin-d_3, was used as internal standard, being added to the samples at the beginning of the analytical workflow prior to sample extraction.

As the analytes possess quite high polarities, liquid chromatography was employed as separation technique in the course of analysis. It was coupled to triple-quadrupole tandem mass spectrometry to obtain highly selective detection and sensitive quantification.

With regard to sample preparation, the honey matrix is characterized by its high sugar content. It is essential that sample extraction and cleanup remove as much of the sugar as possible to avoid contamination problems in the subsequent LC-MS/MS measurement, especially regarding the ion source.

In a first approach a ChemElut type approach,[15] which has been successfully used by others for the analysis of a subset of the neonicotinoids in honey,[11,12] was evaluated. To that end honey spiked at levels of 10 and 100 μg/kg was extracted with methanol/water, cleaned up by liquid−liquid extraction, and measured by LC-MS/MS using solvent standards. The recovery rates obtained ranged from 0 to 149%. Dinotefuran showed low recovery rates with a maximum of 45%, whereas nitenpyram was lost completely. The reason for the substantial losses of dinotefuran and nitenpyram was investigated by inspecting the different steps of the sample preparation method and the analysis by LC-MS/MS. First, extracts of blank honey were spiked with dinotefuran and nitenpyram and analyzed by LC-MS/MS. Recovery rates of ≥100% were obtained for both substances, indicating that matrix effects in the LC-MS/MS were not responsible, but pointing toward substance losses occurring during sample preparation. The liquid−liquid extraction on the ChemElut cartridge was examined by subjecting a nitenpyram standard solution to this step. No nitenpyram could be detected in the eluate, clearly showing that nitenpyram was retained in the ChemElut cartridge. The complete loss of nitenpyram and the partial loss of dinotefuran in the liquid−liquid extraction step can be rationalized by the high hydrophilicities of nitenpyram and dinotefuran expressed by their low log P values of −0.66 and −0.55, respectively.[16] The other neonicotinoids are less hydrophilic, with log P values between −0.24 and 1.26.[16] As a consequence of their high hydrophilicities, nitenpyram and dinotefuran remain completely or to a large extent, respectively, in the methanol−water phase that is adsorbed onto the diatomaceous earth of the ChemElut cartridge and do not partition into the cyclohexane/ethyl acetate eluent.

Consequently, a different sample preparation method had to be found. Thus, in a second approach a QuEChERS type methodology[14] was studied. Honey spiked at three levels (10, 50, and 100 μg/kg) was extracted with acetonitrile, cleaned up by dispersive solid phase extraction, and measured by LC-MS/MS using solvent standards. The achieved recovery rates ranged from 54 to 164%, being clearly superior to those of the ChemElut type method. The improvement was especially significant for nitenpyram, which was completely lost in the ChemElut type approach but exhibited recovery rates ranging from 54 to 79% for the QuEChERS type method. Even though the switch to the QuEChERS type sample preparation had effected a great improvement, recovery rates were still not completely satisfactory. According to the EU validation guideline for pesticide residues, mean recovery values should be within the range of 70−120% at each spiking level.[17] The observed recovery rates result from a combination of losses during sample preparation and of matrix effects in the LC-MS/MS measurements, as clothianidin-d_3 constitutes an "ideal" internal standard only for clothianidin, whereas the other analytes may experience different sample preparation losses and matrix effects.

Table 3. Recovery Rates and Precision Data for All Analytes (n = 5)

	spike level					
	10 μg/kg		50 μg/kg		100 μg/kg	
analyte	RR[a] (%)	RSD[b] (%)	RR (%)	RSD (%)	RR (%)	RSD (%)
acetamiprid	102.1	7.6	89.0	8.2	87.4	4.8
acetamiprid metabolite IM 2-1	99.2	7.5	91.5	7.5	86.6	4.1
clothianidin	99.7	8.1	93.0	3.5	94.3	4.0
dinotefuran	87.3	4.8	83.9	11.8	83.9	9.1
flonicamid	103.5	2.7	94.5	8.1	96.8	6.2
flonicamid metabolite TFNA-AM	114.2	8.2	83.7	9.9	82.9	6.4
imidacloprid	100.7	12.8	101.8	8.9	107.0	7.9
nitenpyram	76.5	7.7	67.3	8.3	60.0	9.2
thiacloprid	98.5	4.1	88.4	8.6	86.3	6.0
thiamethoxam	93.3	9.7	83.5	12.3	82.0	10.1

[a] Recovery rate. [b] Relative standard deviation.

To address the latter, matrix-matched standards were prepared. Subsequent analyses of spiked honey samples with these standards finally showed, in general, very satisfactory recovery rates (Table 3). The recovery rates for nitenpyram were still not optimal due to significant losses during sample preparation, which are caused by its high hydrophilicity, which cannot be compensated adequately by the employed internal standard clothianidin-d_3. A possible solution would be to use an isotopically labeled form of nitenpyram for recovery correction for this analyte. However, such an internal standard was not available. Nevertheless, nitenpyram still achieved recoveries of ≥60% with good reproducibility, which is acceptable.

In the LC-MS/MS determination the chromatographic gradient was optimized to obtain a short run time, an even distribution of the analytes within the elution window, and sufficient retention to avoid elution with highly polar matrix compounds eluting near the void (data not shown). With the final gradient all analytes eluted in a time window between 4 and 13 min, achieving all mentioned aims (Figure 2). The retention times were very stable, with relative standard deviations ranging from 0.17 to 1.43% (n = 10).

Validation. The final method using the QuEChERS type method for sample preparation and matrix-matched standards for quantification was fully validated according to the SANCO/10684/2009 document for method validation and quality control procedures for pesticide residues analysis in food and feed.[17]

Limits of Detection (LODs) and Quantification (LOQs). The determination of LODs and LOQs was based on minimum signal-to-noise ratios of 3:1 and 10:1, respectively. Both SRM traces had to exhibit the required signal-to-noise ratio to ensure unequivocal identification and correct determination of ion ratios. In any case, the "reporting" LOQ was not set below the lowest level of calibration (equivalent to 2 μg/kg) and, hence, the

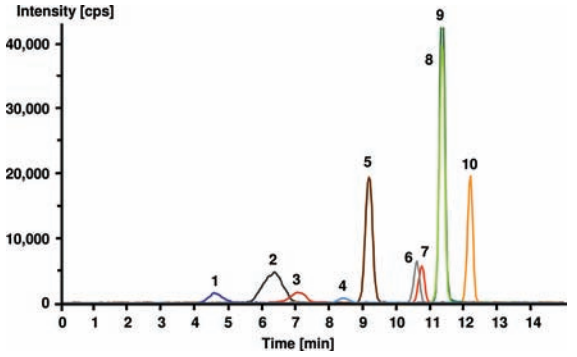

Figure 2. Overlaid LC-MS/MS chromatograms of the investigated analytes. Shown are the traces of the respective first SRM transition: 1, flonicamid metabolite TFNA-AM; 2, dinotefuran; 3, nitenpyram; 4, flonicamid; 5, thiamethoxam; 6, imidacloprid; 7, clothianidin; 8, acetamiprid metabolite IM 2-1; 9, acetamiprid; 10, thiacloprid.

Table 4. Limits of Detection and Quantification for All Analytes Encompassed by the Developed Method

analyte	LOD (μg/kg)	LOQ (μg/kg)
acetamiprid	0.6	2
acetamiprid metabolite IM 2-1	0.6	2
clothianidin	0.6	2
dinotefuran	2	5
flonicamid	2	5
flonicamid metabolite TFNA-AM	2	5
imidacloprid	0.6	2
nitenpyram	5	10
thiacloprid	0.6	2
thiamethoxam	0.6	2

LOD not below 0.6 μg/kg. The LODs and LOQs of all investigated analytes are given in Table 4. Those neonicotinoid insecticides that constitute the active ingredients of plant protection products currently registered in Austria (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam) all had a LOD of 0.6 μg/kg and a LOQ of 2 μg/kg. For dinotefuran, flonicamid, nitenpyram, and the flonicamid metabolite TFNA-AM, the LODs and LOQs were slightly higher, being between 2 and 5 μg/kg for the LOD and between 5 and 10 μg/kg for the LOQ. Overall, the present method can be considered as highly sensitive, allowing the detection and quantification of very low concentrations of neonicotinoid insecticide residues in honey.

Linearity. Calibrations were performed using six levels ranging from 2 to 100 μg/L for all analytes with the exception of nitenpyram. For nitenpyram the calibration was based on five levels (5−100 μg/L). The regression coefficients of the calibration curves were >0.99 for all analytes for both SRM transitions, indicating very good linearity.

Recovery Rates and Precision. According to SANCO/10684/2009, recoveries for all analytes should be within a range of 70−120% for all spiking levels with relative standard deviations of ≤20%. For the determination of the recovery rates and precision, a blank honey was spiked at three concentration levels (10, 50, and 100 μg/kg), and each was analyzed five times. The recovery rates were within the required range for all analytes with the exception of nitenpyram (Table 3), which was partially lost during sample preparation due to its high hydrophilicity. However, the validation guideline allows for recoveries outside the mentioned range providing that suitable reproducibility is achieved. All relative standard deviations were <13% and thus fulfilled the requirement of the SANCO/10684/2009 guideline.

Table 5. Results of the Investigation of Neonicotinoid Insecticides in Flower Honey Samples (Means of Duplicate Analyses)[a]

sample	thiacloprid (μg/kg)	acetamiprid (μg/kg)	thiamethoxam (μg/kg)
A	**26.0**	<LOD	<LOD
B	**27.4**	<LOD	<LOD
C	<LOD	<LOD	<LOD
D	<LOD	<LOD	<LOD
E	<LOD	<LOD	<LOD
F	<LOD	<LOD	<LOD
G	**8.6**	<LOD	<LOD
H	**detectable**	<LOD	<LOD
I	**6.2**	<LOD	<LOD
J	**19.6**	<LOD	**detectable**
K	**11.2**	<LOD	<LOD
L	<LOD	<LOD	<LOD
M	<LOD	<LOD	<LOD
1	<LOD	<LOD	<LOD
2	<LOD	<LOD	<LOD
3	<LOD	<LOD	<LOD
4	<LOD	<LOD	<LOD
5	**5.5**	<LOD	<LOD
6	<LOD	**2.2**	<LOD
7	**5.0**	**15.2**	<LOD
8	<LOD	<LOD	<LOD
9	<LOD	<LOD	<LOD
10	**detectable**	<LOD	<LOD
11	<LOD	<LOD	<LOD
12	**detectable**	<LOD	<LOD
13	**12.3**	<LOD	<LOD
14	<LOD	<LOD	<LOD
15	**detectable**	<LOD	<LOD
16	<LOD	<LOD	<LOD
17	**detectable**	<LOD	<LOD
18	<LOD	<LOD	<LOD
19	<LOD	<LOD	<LOD

[a] All other analytes were <LOD for all samples.

Analysis of Honey Samples. The validated method was employed for the analysis of 41 honey samples collected in different regions of Austria in 2009, consisting of 32 flower honeys (produced from nectar of plants) and 9 forest honeys (produced from excretions of plants or plant-sucking insects). All honey samples were analyzed in duplicate. The main targets of the analyses were to examine the levels of neonicotinoid insecticide residues in Austrian honey and investigate possible differences in residue levels between flower and forest honey samples.

In the 32 flower honey samples only three of the analytes, namely, thiacloprid, acetamiprid, and thiamethoxam, were found (Table 5). In 14 samples thiacloprid was detected, with 9 samples containing residues of this pesticide above the LOQ. The determined

Table 6. Results of the Investigation of Neonicotinoid Insecticides in Forest Honey Samples (Means of Duplicate Analyses)[a]

sample	thiacloprid (μg/kg)
1	<LOD
2	**2.1**
3	<LOD
4	<LOD
5	<LOD
6	**detectable**
7	**detectable**
8	**detectable**
9	<LOD

[a] All other analytes were <LOD for all samples.

quantities of thiacloprid ranged from 5.0 to 27.4 μg/kg. The analyses further showed the presence of acetamiprid in two samples (2.2 and 15.2 μg/kg, respectively) and traces of thiamethoxam in one sample. Two samples contained residues of two neonicotinoids. Of the nine forest honey samples, four contained small amounts of thiacloprid below or at the LOQ (Table 6). No other neonicotinoid residues were observed in these honeys.

Overall, the investigation of neonicotinoid insecticide residues in 41 honey samples from 2009 showed the presence of three neonicotinoids: thiacloprid (18 samples), acetamiprid (2 samples), and thiamethoxam (1 sample). The comparison of the determined concentrations with the MRLs for thiacloprid (200 μg/kg) and acetamiprid (50 μg/kg)[18] showed that all residues were (far) below the respective limits. This clearly indicates the absence of any health risk for the consumer.

The results of the investigated honey samples are in good agreement with recent analyses of the neonicotinoids acetamiprid, clothianidin, imidacloprid, nitenpyram, thiacloprid, and thiamethoxam in honey samples in Germany.[19] In those investigations two neonicotinoids, thiacloprid and thiamethoxam, were detected in the analyzed honey samples. Thiacloprid was found in 75% of the samples in concentrations between 2 and 110 μg/kg, whereas only one honey sample contained traces of thiamethoxam (1 μg/kg).

Throughout the various flower and forest honey samples, the employed method exhibited excellent selectivity, as no interferences in the LC-MS/MS chromatograms were observed (Figure 3). All results above the LOD were successfully confirmed by a second SRM transition, and very good agreements of the SRM ratios of the samples with the reference values calculated from the matrix standards were observed (Figure 3).

By comparison of the different sample categories, the flower honey samples exhibited on average higher amounts of neonicotinoid residues than the forest honey samples. Whereas 10 of 32 samples (31%) contained neonicotinoids above the LOQ for the flower honeys, the same situation was found in only 1 of 9 samples (11%) for forest honeys. This is in good agreement with the different sources of the various sample types.

The species of neonicotinoids for which residues were found link well with their bee toxicities. Thiacloprid was the neonicotinoid that was detected most frequently in the investigated honey samples. It has a relatively low bee toxicity as indicated by an acute oral LD_{50} of 17.32 μg/bee.[20] Thus, honeybees are not easily exposed to lethal doses of thiacloprid during their foraging activities and are thus able to transport thiacloprid into the beehive. This reasoning can also be applied to the findings of

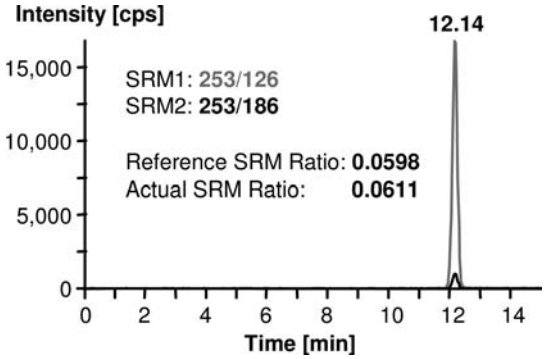

Figure 3. LC-MS/MS chromatogram of a flower honey sample containing thiacloprid. Shown are the two SRM transitions of thiacloprid.

acetamiprid (acute oral LD_{50} = 14.53 μg/bee[20]), which was also detected above the LOQ in some of the analyzed samples. In contrast, only traces of the highly bee-toxic thiamethoxam (acute oral LD_{50} = 0.005 μg/bee[20]) were detected in a single sample and no residues of the even more bee-toxic clothianidin (acute oral LD_{50} = 0.00379 μg/bee[20]). Exposure of foraging honeybees to even relatively small amounts of these highly toxic neonicotinoids is expected to lead to the death of the bees before they can reach the hive and transfer these neonicotinoids into the honey. Another reason for the more frequent detection of thiacloprid compared to the other investigated neonicotinoid insecticides might be the wide and versatile application of plant protection products containing thiacloprid as active ingredient. Thiacloprid is the active ingredient of the widely used plant protection product Biscaya, which is applied as spray in different agricultural crops such as barley, maize, rye, oat, wheat, rape, and potato.[21]

In conclusion, the developed sensitive and selective LC-MS/MS method for the analysis of neonicotinoid residues in honey encompassing all analytes contained in the EU residue definitions for the entire group of neonicotinoid insecticides is a useful tool for the monitoring of honey samples for these substances. The importance of such regular analyses is shown by the detection of residues of three neonicotinoids (acetamiprid, thiacloprid, thiamethoxam) in Austrian honey samples, which confirms the actual occurrence of a transfer of neonicotinoid insecticides from exposed honeybees into honey.

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ABBREVIATIONS USED

LOD, limit of detection; LOQ, limit of quantification; SRM, selected reaction monitoring; PSA, primary−secondary amine.

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