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ABSTRACT

The aim of the study was to provide a comprehensive overview of neonicotinoid pesticide residues in honey samples for a single country and compare the results with the import data for neonicotinoid pesticides. The levels of four neonicotinoid pesticides, namely thiamethoxam, imidacloprid, acetamiprid, and thiacloprid, were determined in 294 honey samples harvested from 2005 to 2013 from more than 200 locations in Estonia. For the analyzed honey samples, 27% contained thiacloprid, and its levels in all cases were below the maximum residue level set by the European Union. The other neonicotinoids were not detected. The proportion of thiacloprid-positive samples for different years correlates well with the data on thiacloprid imports into Estonia, indicating that honey contamination with neonicotinoids can be estimated based on the import data.

Introduction

Neonicotinoids, such as imidacloprid and acetamiprid, are a relatively new class of insecticides. Neonicotinoids affect the nervous system of the insects through nicotinic acetyl choline receptors. These pesticides are widely used because they act as strong agonists, activating the nicotinic acetyl choline receptors of insects. However, the effect is not as significant for vertebrates. They are therefore highly toxic for insects, but are generally considered only moderately harmful to vertebrates. [1–3]

Neonicotinoids are suspected as possible contributors to the sudden decline of the bee population in recent decades due to the so-called colony collapse disorder,[4,5] which is believed to be caused by the concurrence of different factors.[6] The specific mechanism by which neonicotinoids are a cause of colony collapse disorder is still to be identified, but several possible pathways have been proposed, including increased susceptibility to pathogens or parasites by weakened immunity,[5] a negative impact on the reproduction of bees,[7] or increased mortality from homing failure.[8]

A recent paper by Kimura-Kuroda et al.[9] revealed the potential danger of two neonicotinoids (imidacloprid and acetamiprid) to the development of the human nervous system. A recent report by the European Food Safety Authority (EFSA) confirmed the potential danger to humans, and proposed a decrease of the acceptable levels for the acute reference dose and acceptable operator exposure level for both compounds and acceptable daily intake of acetamiprid. In addition, the report called for submission of mandatory developmental neurotoxicity studies as part of the pesticide authorization process.[2]

Besides determination of the exposure of bees to neonicotinoids, it is increasingly important to determine neonicotinoids in products consumed by humans. This is illustrated by a study published in 2014 on the analysis of 573 fruit and 850 vegetable samples collected randomly from a market in the Aegean region of Turkey between 2010 and 2012. From 186 pesticide residues determined in the study, one of the three most frequently detected pesticides was acetamiprid (in over 20 fruit and over 120 vegetable samples). In addition, imidacloprid was also detected frequently (in over 20 fruit and over 40 vegetable samples). Thiamethoxam was observed less frequently.[10]

Determination of neonicotinoids in honey serves both of these purposes by giving an idea as to what extent bees are carrying neonicotinoids in honey. It is necessary to ensure that the levels of neonicotinoids in honey are below the maximum residue levels (MRLs) set by European Union (EU) (Reg. No. 364/2014 (thiacloprid), 500/2013 (acetamiprid), and 893/2010 (imidacloprid)). According to Reg. (EU) No. 485/2013, a two-year restriction for seed treatment, soil application, and foliar treatment on plants and cereals with three neonicotinoids (clothianidin, imidacloprid, and thiamethoxam) in the EU has been established. Therefore, it is important to have an overview of the previous levels of neonicotinoids in honey in EU countries for observation of the changes that will have due to the restrictions.

Neonicotinoids have been determined in many different matrices,[11–13] including honey samples, by geographical location or year-wise, in different countries. Among other pesticides, thiamethoxam and imidacloprid were analyzed in 45 samples collected from northern Poland in 2010. Overall, 11% of the samples were positive for both of these pesticides (thiamethoxam was also found over the MRL).[14] Thiacloprid, acetamiprid, thiamethoxam, and imidacloprid were analyzed in 41 honey samples collected in 2009 from different regions of
Austria. All neonicotinoids, besides imidacloprid, were detected. Thiacloprid was most frequently detected, occurring in 18 samples. The obtained concentrations were below the MRLs.15 In a study carried out in France, honey samples were collected for every two weeks from April to August in 2009 and 2010 from two apiaries, giving a total of 90 samples. From the samples, 22 insecticides were analyzed. From the analyzed insecticides, neonicotinoids, thiacloprid, thiamethoxam, and acetamiprid were detected. Acetamiprid concentrations ranged over the MRL.16 Lambert et al.17 analyzed 80 pesticides (including imidacloprid and thiamethoxam) in 141 honey samples collected from western France. Imidacloprid was only found in three honey samples, and thiamethoxam was not found in any of the samples.17 No neonicotinoids were found in 14 honey samples from Greece (2011–2013), although imidacloprid, thiamethoxam, and thiacloprid were found in some honeybee samples.18 Analysis of 104 samples from the Autonomous Province of Vojvodina, Serbia, found thiacloprid in five samples, imidacloprid in four samples, and thiamethoxam in five samples, all below the MRLs.19 In summary, a number of studies on neonicotinoids in honey have been carried out but only a few studies have a considerable number of samples over an extensive time period. According to our knowledge, no data from northern European countries have been published.

Neonicotinoids have been determined in honey mainly by the liquid chromatography–electrospray-mass spectrometry (LC/ESI/MS) method20–23 and also by the enzyme-linked immunosorbent assay technique.24 Honey is essentially a mixture of water, monosaccharides, and oligosaccharides containing different compounds. Their proportions depend on the nature of water, monosaccharides, and oligosaccharides containing different compounds. Their proportions depend on the

The aim of this work was to determine four neonicotinoids, namely imidacloprid, acetamiprid, thiamethoxam, and thiacloprid, in a large number of honey samples collected over a time period of nine years, to give an overview of the neonicotinoid pesticide levels in honey in Estonia, a EU country located in northern Europe. There is currently no overview of the levels of neonicotinoids in honey in Estonia, or indeed in any other country of northern Europe. Also, to the best of our knowledge, a study of neonicotinoids in honey that is equally comprehensive in terms of both sample numbers and time-scale has not been yet been published for a single country.

**Materials and methods**

**Chemicals**

Standards for thiamethoxam (99.0%, 3-[(2-chloro-1,3-thiazol-5-yl) methyl]-5-methyl-N-nitro-1,3,5-oxidazinan-4-imine), imidacloprid (99.5%, 1-[(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylidineamine], acetamiprid (98.5%, (E)-N-[6-chloro 3-pyrrolil)methyl]-N^5-cyano-N^1-methylacetamidine), and thiacloprid (98.0%, (Z)-3-(6-chloro-3-pyridylmethyl)-1,3-thiazolidin-2-ylide

**Instruments**

Analysis of honey samples was performed on an Agilent Series 1100 LC/MSD Trap XCT (Agilent Technologies, Santa Clara, CA, USA). The LC instrument was equipped with a binary pump, an autosampler, and a thermostated column compartment. The injection volume was 5 μL. The mass spectrometer used a quadrupole ion trap mass analyzer. For instrument control, an Agilent ChemStation for LC Rev. A. 10.02 and MSD Trap Control version 5.2 were used. The ion transportation parameters were optimized for each analyte at a chromatographic flow rate via MSD Trap Control software. For ESI, the 3R nebulizer, introduced by Kruve et al., was used with the pressure of the nebulizer gas set to 2 psi (minimum value) and the drying gas set at 10 L min⁻¹ with a temperature of 350°C; the gas pressure of the additional nebulizing capillary inside the liquid capillary was set to 14 bar.

For separation, a 250-mm long Zorbax Eclipse XDB-C18 column with an Eclipse XDB-C18 12.5-mm pre-column (both with an internal diameter of 4.6 mm and a particle size of 5 μm) was used. Gradient elution (flow rate 0.8 mL min⁻¹) was used with acetate buffer and methanol. The methanol percentage (v/v) was raised from 40 to 100% in 17 min, maintained at 100% for 5 min, and lowered back to 40% in 3 min. The stabilization time between runs was 7 min.

For sample pretreatment, a centrifuge (Centrifuge 5430R) and stirrer (MixMate from Eppendorf (Hamburg, Germany)) were used.

**Sample pretreatment**

The honey samples were collected between 2005 and 2013, and were provided from different sources, thus different storage conditions have been applied. The majority of honey samples, 141, were the same as used by Rebane and Herodes. Samples obtained from the Estonian Environmental Research Centre (EERC) and 39 samples from Estonian beekeepers.

Samples used by Rebane and Herodes were mostly stored at room temperature since their collection from 2005–2010, samples from the EERC (2010–2013) were kept in a dark room, designated only for honey samples with the temperature kept from 10-17°C. Honey sample collection was completed by one person from markets, stores, and fairs in their original
packages. After acquiring the samples directly from beekeepers in January 2014 (collected by beekeepers mostly in the summer of 2013, with some samples from 2011 and 2012), they were kept in a fridge at −20°C.

For sample pretreatment, the modified QuEChERS method was used. Honey (1 g) was dissolved in 10 mL of purified water and 10 mL of acetonitrile. MgSO₄ (4 g) and NaCl (1 g) were added and shaken for 1 min, followed by centrifugation for 3 min at 4400 rpm. An acetonitrile fraction of 1 mL was pipetted into a 2-mL centrifuge tube with 150 mg of MgSO₄ and 25 mg of PSA for cleanup, followed by stirring for 1 min. Tubes were centrifuged for 1 min at 5000 rpm, and the supernatant was taken for analysis. For every honey sample, sample pretreatment was performed, followed by subsequent analysis on the same day.

Validation

The method was validated according to the SANCO guidelines (SANCO/12571/2013). Neonicotinoids were baseline separated; an extracted ion chromatogram showing the separation of the four neonicotinoids is presented in Figure 1. The limit of detection (LOD) calculation method was taken from the ICH harmonized tripartite guidelines, 2005. The linearity of the method was estimated using spiked extracts of a polyfloral honey sample in a concentration range of 390 ng kg⁻¹ to 15.6 mg kg⁻¹ (at least 10 points were used for each analyte). The linear range was determined according to residual analysis (both absolute and relative with overlapping area as a linear range), as recommended by Kruve et al.

Repeatability (RSD) and process efficiency (PE) were estimated based on three different honey samples with five replicates. PE accounts for recovery from the sample pretreatment and also for matrix effects, and therefore characterizes the overall efficiency. Honey samples used in the validation were polyfloral honey, dandelion honey, and rapeseed honey. The higher concentrations used for the RSD analysis for thiamethoxam, imidacloprid, acetamiprid, and thiacloprid were 1.6, 0.26, 0.13, and 0.065 mg kg⁻¹, respectively. The low concentrations used for the RSD analysis for thiamethoxam, imidacloprid, acetamiprid, and thiacloprid were 0.39, 0.0624, 0.0195, and 0.0078 mg kg⁻¹, respectively.

The LOD was determined using the standard deviation of the calibration graph residuals in the LOD region (ICHguidelines), as recommended by Kruve et al.: LOD = 3.3 × σ S⁻¹, where σ is the residual standard deviation and S the slope of the calibration graph. Five to seven points for the graph were chosen to cover one order of magnitude of concentration, starting from the lowest used concentration. The limits of quantification (LOQ) was determined by LOQ = 10 × σ S⁻¹.

At first, selectivity was ensured by monitoring two ion transitions. For thiamethoxam, the parent ion was 314 and the monitored fragments were 210 and 180, for imidacloprid, 256 → 209 and 175, for acetamiprid, 223 → 126 and 187, and for thiacloprid 253 → 126 and 186 (Table 1). After finding positive samples, an additional third ion transition for confirmation was used (thiacloprid 253 → 226). Samples where all three ions could not be detected were assigned as negative. Additional confirmation of positive samples was achieved by monitoring the abundance ratio of the two most intense fragments. The acceptable boundaries for the abundance ratio were calculated from 64 calibration samples from eight days with a concentration range of LOD up to 0.3 mg kg⁻¹. The acceptable ratio was found as the mean ratio ± two standard deviations of the ratio found in calibration samples. Analysis of positive samples was repeated with the same criteria. In Figures A1–A4 in Appendix, the proposed fragment compositions are provided for all neonicotinoids.

Table 1. Comparison of limits of quantification (LOQs), linear ranges, repeatabilities (RSDs), and process efficiencies (PEs) together with MRL values. Parent ions with quantification fragments and identification fragments are also shown.

<table>
<thead>
<tr>
<th>m/z transitions</th>
<th>Parent ion</th>
<th>Fragments</th>
<th>LOD (mg kg⁻¹)</th>
<th>LOQ (mg kg⁻¹)</th>
<th>MRL (mg kg⁻¹)</th>
<th>Linear range (mg kg⁻¹)</th>
<th>RSD (%)</th>
<th>PE (%)</th>
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</thead>
<tbody>
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<tr>
<td>Thiamethoxam</td>
<td>314</td>
<td>210, 180</td>
<td>0.10</td>
<td>0.31</td>
<td>NA</td>
<td>0.31–2.0</td>
<td>5.5</td>
<td>12</td>
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<tr>
<td>Imidacloprid</td>
<td>256</td>
<td>175, 209</td>
<td>0.018</td>
<td>0.055</td>
<td>0.05</td>
<td>0.055–0.31</td>
<td>5.3</td>
<td>12</td>
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<tr>
<td>Acetamiprid</td>
<td>223</td>
<td>126, 187</td>
<td>0.0056</td>
<td>0.017</td>
<td>0.05</td>
<td>0.017–0.16</td>
<td>9.9</td>
<td>13</td>
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</tr>
<tr>
<td>Thiacloprid</td>
<td>253</td>
<td>126, 186, 226</td>
<td>0.0014</td>
<td>0.0043</td>
<td>0.2</td>
<td>0.0047–0.16</td>
<td>5.4</td>
<td>14</td>
</tr>
</tbody>
</table>

*Na adduct; *quantitation ion; *high/low conc., high/low concentration end of linear range; *MRL for thiamethoxam (0.01 mg kg⁻¹) is given as a sum of thiamethoxam and clothianidin.
at room temperature for 10 months. This experiment enables the estimation of the stability of thiacloprid in honey and was not used for quantitation. A \( t \)-test at 95% confidence intervals (CI) was used to test whether the concentration of thiacloprid was significantly influenced by storing conditions.

For honey samples containing thiacloprid, proportions with 95% CI were calculated on a year-wise basis using the following formula:

\[
p = \frac{n_{\text{pos}} + 2}{n + 4}; \quad W = 2 \times \sqrt{\frac{p(1-p)}{n + 4}}.
\]

where \( p \) is proportion, \( n_{\text{pos}} \) is the number of samples containing thiacloprid, \( n \) is the overall number of samples, and \( W \) is the error margin at a 95% CI.[34]

Results

The LOD values for thiamethoxam, imidacloprid, acetamiprid, and thiacloprid were 102, 18, 5.6 and 1.4 \( \mu \text{g kg}^{-1} \), respectively. The LOQ, linear range, repeatability (expressed as RSD), and PE values for thiamethoxam, imidacloprid, acetamiprid, and thiacloprid are given in Table 1, together with the MRL values set by Reg. (EU) No. 364/2014 (thiacloprid), 500/2013 (acetamiprid), or 893/2010 (imidacloprid). In Appendix, RSD (Fig. A5) and PE (Fig. A6) with 95% CI value are given.

Among the 141 samples used by Rebane and Herodes,[29] there were samples of mono- and polyfloral honey from different parts of Estonia, providing a representative selection of honey collected from 2005–2010. Of the 141 samples, 11 (8%) contained thiacloprid at the LOD level. Thiamethoxam, imidacloprid, and acetamiprid were not detected in any of the samples. Of the 114 samples obtained from the EERC (2010–2013) and the 39 samples (mostly in 2013) obtained from Estonian beekeepers 48 (42%) and 20 (51%) samples, respectively, contained thiacloprid at the LOD level or above the LOQ level. The geographical distribution of the samples acquired and the percentage of samples containing thiacloprid are shown in Figure 2. The proportions (with 95% CI) of samples containing thiacloprid are shown year-wise in Figure 3.

In Table 2, the thiacloprid concentrations in samples over the LOQ are reported year-wise. In addition, floral composition is reported where data were available. In the case of samples acquired directly from beekeepers, major floral gathering sites for honeybees are presented.

Results from the \( t \)-test gave no statistically significant differences between the replicates of honey samples stored under different conditions for the same time (room temperature vs. \(-20^\circ\text{C}\) and room temperature vs. \(40^\circ\text{C}\)).
It is also interesting to note that the observed fragment ions were different for thiamethoxam from those commonly reported in the literature (Fig. 4). In the literature, the common fragments, corresponding to the thiamethoxam molecular ion ([M+H]+), are 211 and 181. In this work, thiamethoxam was observed as a Na+ adduct with fragments 210 and 180 and the [M+H]+ ion was much less intense. This Na+ adduct effect was studied on two different MS instruments (the used ion-trap and also on an Agilent 6495 Triple Quad LC/MS/MS instrument with a multimode ionization source). While on the triple quadrupole instrument, the [M+H]+ ion for thiamethoxam (with observed fragments 211, 181, 210, and 180) was more intense than [M+Na]+, the effect was the same as observed on the ion-trap instrument, that is fragments 211 and 181 were absent and 210 and 180 were most intense. Thus, the observed fragments are suitable for detection of thiamethoxam.

**Discussion**

**Validation**

A literature overview shows that the LODs for neonicotinoids in honey have been 0.15–160 µg kg⁻¹ for thiamethoxam, 0.03–33 µg kg⁻¹ for imidacloprid, 0.04–0.5 µg kg⁻¹ for acetamiprid, and 0.02–0.5 µg kg⁻¹ for thiacloprid.[14,20–23,35] While the values for thiamethoxam and imidacloprid obtained in this work are within the range found in the literature for acetamiprid and thiacloprid, these are higher. However, the LOD and LOQ values are strongly dependent on the instruments used, as well as on the approach used for their determination. The approach recommended by Kruve et al.[33] is known to give, on purpose, very conservative LOD values, significantly higher than the LOD estimates based on signal-noise ratio. The reason for such conservative LOD values is that at the LOD level, the method in a laboratory must be safely able to detect the analyte on different days.[33] It is thus not clear how much the detection capability of the current method is different from the average level reported in the literature.

In Table 1, the linear ranges, LOQ, and MRL values are shown. Comparison of LOQ values with the MRL values shows that both acetamiprid and thiacloprid LOQ values were below the MRL values, and the LOQ for imidacloprid was at par with the MRL value. As can be seen in Table 1, the linear range extends to MRLs for all the neonicotinoids except thiamethoxam.

According to SANCO/12571/2013, the RSD values should be below 20%. As can be seen in Table 1, the pooled RSD values were below the suggested 20%. From Figure A5 in Appendix, it

![Figure 4. Thiamethoxam-proposed fragmentation scheme.](image-url)
can be seen that only the upper confidence interval for thiaco-
prid at the low concentration end of the linear range is slightly
over 20%. Also, the recovery values should be between 70% and
120%. In the context of LC/ESI/MS, it is appropriate to com-
pare these values with PE, not to recovery, because PE also
takes into account possible ionization suppression. For three of
the neonicotinoids, thiamethoxam, imidacloprid and thiaco-
prid, the PE values with 95% CIs were within recommended
limits. For acetamiprid, the average PE with confidence inter-
vals was not within the recommended limits (Fig. A6). This,
however, was not a problem in the current analysis, since acet-
amiprid was expected at low concentrations (if at all).

Considering the results from the validation, the method has
been shown to be fit for analysis of the four neonicotinoids in
honey.

Neonicotinoids in honey samples

From the 294 Estonian honey samples analyzed, 27% contained
thiacloprid either at or above the LOD level. In addition, 23
samples from Eastern Finland were analyzed, which were col-
lected from an area rich in common heather (Calluna vulgaris)
and rosebay willowherb (Epilobium angustifolium) with little or
no agriculture. All of these 23 samples were free of all four
neonicotinoids. This serves as a validity test for the method to
confirm that no false positive results were obtained.

In Figure 2, the geographical distribution of positive samples
(confirmation was carried out based on three fragment ions and
their intensity ratio) is shown. Proportion analysis at 95% CIs
revealed that statistically significant difference was found for
Jõgeva county (with a high proportion of 80% positive samples)
with Hiiumaa (7%), Saare (0%), and Pärnu (12%) counties. This
means that the latter three counties have statistically significant
lower positive sample percentages than Jõgeva county. How-
ever, no statistically significant differences were observed in
comparison with other counties. Of the samples, 44 did not
have information about the collection location specified (30%
of these samples contained thiacloprid) and are thus not shown
on the map. Also, no correlation between the percentage of
positive samples and the percentage of agricultural land in a
county (data from 2010 by area and arable land) was observed
(R² = 0.24) (Table A1 in Appendix).[35,36]

Samples containing thiacloprid were not equally distributed
between 2005 and 2013. For samples from 2005 to 2007, no
neonicotinoids were detected. In Figure 3, however, the propor-
tions from 2005 to 2007, calculated according to Eq. (1), are not
zero because of the nature of Eq. (1). The rationale behind this,
seemingly odd result, is that there is an insufficient number of
samples for claiming a zero proportion. In fact, it is more prob-
able that even though in this investigation positive samples
were not detected, they may actually exist. The number of sam-

dles containing thiacloprid from 2008 to 2013 were 3 (out of 56
samples), 7 (25), 10 (33), 10 (31), 19 (32), and 30 (out of 60
samples), respectively. An increasing trend can be seen through
the years (seen also in Fig. 3 based on proportions with 95%
CIs). This can either be because the pesticides containing thiaco-

prid were not so widespread in earlier years or thiacloprid
has partially decomposed in the older honey samples during
storage, or both.

According to the Estonian Agricultural Board database,[38]
the use of pesticides containing thiacloprid started in 2004
(Fig. 3). An increasing trend of imports correlates with the
increasing number of positive samples. There is a sharp
increase in imports of thiacloprid in 2007, matching the detec-
tion of thiacloprid in a large number of honey samples from
2007. The correlation suggests that increase in thiacloprid
import was followed by increase in the number of thiacloprid
containing honey samples, thus providing a robust means of
comparing the expected accumulation of thiacloprid in honey
through years and by country.

The stability of thiacloprid in honey was studied, as this
aspect can affect interpretation of results. According to the sta-
bility tests, thiacloprid concentrations in different honey sam-
dles did not change during the period of 10 months. This
suggests that the amount of thiacloprid in honey samples is sta-
 ble for extended periods, and results obtained for recent years
are comparable. It has been shown that thiacloprid is stable
under environmental conditions (six or more months).[39] The
stability tests and results suggest that thiacloprid is stable in
honey for years, explaining the reason for detecting thiacloprid
in samples as old as five years. It can also be assumed that the
results for recent years are comparable.

From the Agricultural Board database for 2002–2010,[38] it
can be seen that besides thiacloprid, imidacloprid and thiam-
ethoxam were also imported in substantial quantities. These two
neonicotinoids were not detected in honey samples. This could
be because (1) their use was less widespread and in smaller
amounts than thiacloprid as import numbers suggest; (2) they
are less stable than thiacloprid in honey samples; (3) honeybees
that were contaminated with thiacloprid had a higher chance
to reach their hive due to the lower toxicity of thiacloprid in
honeybees compared to imidacloprid and thiamethoxam,[40] or
(4) these two neonicotinoids were not applied on plants that
were providing nectar for honey. The fourth possibility seems
unlikely because neonicotinoids are applied for the protection
of a diverse range of plants (rapeseed, potatoes, sugar beet,
wheat, oat, tomatoes, cucumber, and strawberry) in Estonia.

In the case of thiamethoxam, another possibility is that it
was fully converted to clothianidin in honey. Clothianidin stan-


dard was not available and this compound was not included in
the study.

Of the 294 honey samples analyzed, 19 contained thiaclo-
prid over the LOQ level, up to 0.13 mg kg⁻¹ (the MRL for thia-
cloprid is 0.2 mg kg⁻¹). Year-wise, there were two samples in
2010 that contained thiacloprid over the LOQ level (6% of sam-
ple from 2010), 4 from 2011 (13%), 5 from 2012 (16%), and 8
from 2013 (13%). The increasing trend of positive samples and
the increasing concentrations were also observed in the case of
samples over the LOQ level.

It is noteworthy that all samples containing thiacloprid over
the LOQ level have brassicaceae as part of floral composition.
Floral composition for four samples was not available, but bee-
keepers reported gathering sites with rapeseed (Brassica napus),
which belongs to the brassicaceae family. This is in agreement
with the use of thiacloprid containing pesticides for rapeseed
protection in Estonia (pesticides Proteus OD and Biscaya).[41]
Of the 114 samples from the EERC, only nine were without
brassicaceae and none of these samples contained thiacloprid.
It is likely that over the LOQ level, samples had more exposure to thiacloprid because the honey largely originates from rapeseed fields. Most of the positive samples where thiacloprid concentration was below the LOQ level were reported as polyfloral. However, there were also positive samples reported to have more diverse gathering sites such as common heather (*Calluna vulgaris*) and no rapeseed fields, leaving open other possibilities of honey contamination with thiacloprid than through nectar or pollen gathered from rapeseed fields. One such way can be contamination of groundwater with thiacloprid, resulting in the spreading of thiacloprid farther than the target plant field. Subsequent uptake of thiacloprid by non-target plant can thus expose honeybees to neonicotinoids.

For the samples acquired directly from beekeepers (39 samples), additional information was also available about the well-being of honeybees. There were five cases of unusual increase in mortality in winter where no hives survived or an unusually high number of hives did not survive. In all these samples, thiacloprid was present at slightly above the LOD level or not at all. However, for samples that contained thiacloprid above the LOQ level, no unusual increase in mortality or colony collapse disorder was reported. This was also the case for most of the samples with thiacloprid at residue level. Thus, no correlation between thiacloprid contamination and honey bee mortality could be drawn based on these data.

**Conclusions**

Of the 294 Estonian honey samples analyzed, 27% contained thiacloprid but not over the current EU MRL. No residues of thiamethoxam, imidacloprid, or acetamiprid were detected. The proportion of positive honey samples correlated with the year-wise increase in thiacloprid importation. It is also clear that thiacloprid has a long decomposition time and can be found as residues in honey samples for as long as five years or more. Samples with thiacloprid content over the LOQ level had *brassicaceae* as a common constituent based on either pollen analysis or information from beekeepers about gathering sites (rapeseed fields). To the best of our knowledge, this is the most wide-ranging study of the occurrence of neonicotinoids in honey samples available for a single country in terms of both the number of samples and the time period covered.

**Funding**

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**References**


Appendix

Figure A1. Proposed fragments for thiamethoxam.
Figure A2. Proposed fragments for imidacloprid.

Figure A3. Proposed fragments for acetamiprid.

Figure A4. Proposed fragments for thiacloprid.
Table A1. Comparison of arable area ratio to county area with thiacloprid containing honey samples by county.

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<th>Arable area, ha</th>
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<th>Arable area ratio to county area (%)</th>
<th>Thiacloprid containing honey samples by county (%)</th>
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Figure A5. Comparison of RSD values for all analytes with ESI nebulizers used. Error bars denote 95% CI. Suggested RSD values by SANCO are shown as lines at 20%. THC – thiacloprid, ACA – acetamiprid, IMC – imidacloprid, THM – thiamethoxam.

Figure A6. Comparison of process efficiency values for all analytes with ESI nebulizers used. Error bars denote 95% CI. Suggested process efficiency values by SANCO should be between lines at 70% and 120%. THC – thiacloprid, ACA – acetamiprid, IMC – imidacloprid, THM – thiamethoxam.