LC–MS/MS Analysis of Five Neonicotinoid Pesticides in Sheep and Cow Milk Samples Collected in Jordan Valley

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Giorgio Fedrizzi[1] · Alberto Altafini[2] · Sara Armorini[2] · Khaled Mefleh Al-Qudah[3] · Paola Roncada[2]

[1]*Reparto chimico degli alimenti, Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna "Bruno Ubertini", Via P. Fiorini 5, 40127 Bologna, Italy*

[2]*Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra 50, 40054 Ozzano dell'Emilia (BO), Italy*

[3]*Department of Veterinary Clinical Sciences, Jordan University of Science and Technology, Irbid 22110, Jordan*

Corresponding author: Paola Roncada

Phone number +390512097511 Fax +39051799511

E-mail addresses: giorgio.fedrizzi@izsler.it (G. Fedrizzi), alberto.altafini@unibo.it (A. Altafini), saraarmorini@hotmail.it (S. Armorini), alqudah@just.edu.jo (K. M. Al-Qudah), paola.roncada@unibo.it (P. Roncada).

**Abstract** The purpose of the present study was to evaluate the presence of five neonicotinoid pesticides, acetamiprid, imidacloprid, clothianidin, thiacloprid, and thiamethoxam, in sheep and cow milk samples collected from animals bred in the Jordan Valley. In this area, numerous citrus plantations are present, and these insecticides are commonly used to protect plants from pests and diseases. Thirty-seven sheep milk samples and thirty-one cow milk samples were analysed. The analytical method, based on a single cleanup extraction step with SPE cartridges packed with diatomaceous earth material, together with analysis by LC-MS/MS, has guaranteed average recoveries between 75.1% and 88.3%, limits of detection (LOD) and quantification (LOQ) of 0.5 and 1 µg/kg, respectively, for all the five neonicotinoids. LOQ was much lower than the Codex Maximum Residues Limits (MRLs) for these pesticides in milks. No residues of the five neonicotinoids were found in any sample at a concentration level above LOD.

***Keywords*** *Neonicotinoids · Milk · Jordan Valley · Pesticide residues · Environment · LC-MS/MS*

Neonicotinoid pesticides were introduced in the early 1990s and since then their use globally has expanded rapidly (Limay-Rios et al. 2016; Wood and Goulson 2017). In fact, nowadays, they account for approximately one third of the world insecticide market. Furthermore, they are an integral component in numerous pest and integrated pest management (IPM) strategies (Jeschke et al. 2013; Simon-Delso et al. 2015). They are used on over a hundred different crops for the control of sucking insect pests, but also to control fleas in companion animals and cattle, and to kill cockroaches, houseflies and termites that infest buildings (Elbert et al. 2008; Vo et al. 2010; Jeschke et al. 2011). This class of pesticides is highly versatile in terms of application methods. They can be used with a lot of different application techniques as foliar, seed treatment, soil drench, soil and/or stem injection, and stem application (Jeschke et al. 2011, 2013). In particular, they are highly suitable for use against insects feeding on foliage or plant sap. This is because neonicotinoid pesticides can reach the feeding sites of insects after foliar application through translaminar movement and after soil application through systemic movement from roots to foliage (Van Timmeren et al. 2011). However, the systemic nature and the long persistence increase the probability of environmental contamination and exposure of non-target organisms. This contamination can occur via spray drift from aerial spraying of pesticides, contamination and accumulation in soil and water runoff (Bonmatin et al. 2015). Furthermore, also the leaf litter from treated plants can be a source of contamination of soil and water. Because of the adverse effects of these pesticides on pollinator species, from 2013 the European Commission has imposed restrictions on the use of three neonicotinoids, clothianidin, imidacloprid, and thiamethoxam (De Belder 2018).

Since the climate of Jordan is of arid/semi-arid type, the agricultural activities are largely concentrated in the Jordan Valley, where an intensive irrigated agriculture has been developed. In these areas, trees crop as citrus, olives, bananas, grapes, dates, and seasonal crop as barley, wheat, and vegetables have been planted. Among these crops, citrus trees are the most represented type of cultivation (Venot et al. 2007). Neonicotinoid insecticides are commonly used to treat citrus orchards with foliar spray and via trunk or soil injection for their translaminar and systemic properties. The Valley is also a pasture land, because the grass of the area is grazed by farmed animals or used as feedingstuffs. In particular, sheep graze on it from spring to autumn, and cows bred in the area of Al-Mafraq are fed with harvested fresh grass. Considering the closeness between the citrus trees-growing areas and the pastures, it is reasonable to suppose that sheep and cows can be exposed to neonicotinoid pesticides through ingestion of the grass of the valley.

Humans are exposed to neonicotinoid pesticides through consumption of vegetables or food of animal origin contaminated with the parent compounds or their metabolites (Simon-Delso et al. 2015). In plants and environmental media, neonicotinoids are subjected to intense biotransformation leading to the appearance of different metabolites which can themselves be toxic. These compounds can persist, and possibly accumulate, for month or years. Their metabolism may continue in the animal organisms leading to a wide range of breakdown products (Bonmatin et al. 2015).

It has been shown that neonicotinoid insecticides can concentrate into the milk of food-producing animals after oral intake (FAO 2006; FAO 2008; FAO 2010a, b; FAO 2011). Since milk is a possible way of human exposure to neonicotinoids, maximum residue levels (MRLs) have been established by the Codex Committee on Pesticide Residues (CCPR) to safeguard consumer health. Jordan is a

membership of the Codex Alimentarius Commission since 1966 (Codex Alimentarius), and adopted Codex MRLs as a reference (Khraishy 2017).
It is therefore interesting and important to monitor the milk produced by sheep and cows fed with the grass of the Jordan River Valley to assess if the levels of these contaminants are below the MRLs or, on the contrary, they represent a real risk for the local population. Little information is still available on the possible neonicotinoid residues in food of animal origin and subsequent oral exposure to humans, despite these pesticides have been on the market for over twenty years. Moreover, several scientific papers have been published regarding the quantitative analysis of neonicotinoids in fruit, vegetables, and honey, but very few publications are focused on the quantitative determination of these contaminants in milk.
The aim of this study was to assess the presence of five neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam) in sheep and cow milk samples collected from animals bred in the Jordan Valley area and feeding on contaminated grass. The present study also evaluated the efficiency of the analytical method used to determine these pesticides, which was based on a single cleanup extraction step with SPE cartridges, together with analysis by LC-MS/MS.

# Materials and methods

A total of 68 samples consisting of raw sheep's milk (n=37) and raw cow's milk (n=31) were analysed. Sheep milk samples were collected in the Jordan Valley in the period from April to May 2015 from livestock of five itinerant flocks. Cow milk samples were collected in five farms located in the area of Al-Mafraq in March 2015. Sheep were fed on the pastures of the Jordan River Valley, and cattle were mainly fed with fresh grass harvested in the Valley and with a small amount of straw and silage. Aliquots of 100 ml of milk samples were collected from each animal and kept at 4 °C. The samples were then freeze-dried and stored at -20 °C until analysis. The analytical standards of neonicotinoid pesticides including acetamiprid, clothianidin, imidacloprid, thiacloprid, and thiamethoxam were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Acetonitrile and water (HPLC grade) and dichloromethane (analytical grade) were supplied from Mallinckrodt Baker B.V. (Deventer, The Netherlands). Formic acid (98-100% pure) was obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Chem Elut SPE cartridges packed with diatomaceous earth material were purchased from Agilent Technologies (Santa Clara, CA, USA). Standard stock solutions of each neonicotinoid compound were prepared at 1000 µg/ml by dissolving 10 mg of each standard in 10 ml of acetonitrile. A 100 µg/ml multicomponent stock solution was then prepared by mixing equal volumes of stock solutions of each compound, followed by 1:2 dilution with acetonitrile. Finally, an aliquot of this solution was further diluted (1:10) to obtain a 10 µg/ml multicomponent solution. Solutions at different concentration (0.25, 0.5, 1, 2.5, 5, 10, and 50 ng/ml) were obtained by diluting with methanol in order to prepare calibration curves of the five neonicotinoids. The standard solutions were stored at 5±3 °C and protected from light.
Analysis were performed by LC–MS/MS on an Alliance HT 2695 (Waters, Milford, MA, USA) coupled to a Quattro Ultima Platinum triple-quadrupole mass spectrometer with electrospray ionization source (Micromass, Manchester, UK). The analytical column was a Luna C18(2) 100Å 2x100 mm 3 µm (Phenomenex, Torrance, CA, USA). Chromatographic separation was achieved in gradient conditions and at room temperature. The mobile phase consisted of deionized water (solvent A), and acetonitrile (solvent B), both containing 0.2% formic acid. The elution gradient used was set as follows: from 90% solvent A and 10% solvent B to 100% solvent B in 10 min with linear increase. The total run time was 12 min. The flow rate of the mobile phase was 0.25 ml/min, and the injection volume was 10 µl. Based on the structural properties of analytes, the positive ionization modes (ESI+) was applied. The parameters were as follows: cone voltage, 50V; capillary voltage, 3.8 kV; source temperature, 120 °C; and desolvation temperature 350 °C. Qualitative and quantitative data analysis were performed using Mass Lynx TM 4.0 SP4 software (Micromass, Manchester, UK). The data

acquisition was in multiple reactions monitoring (MRM) mode. The ion transitions and mass parameters monitored for each analyte are reported in Table 1.

**Table 1** Mass spectrometric parameters for the simultaneous determination of 5 neonicotinoids using an electrospray interface (ESI) in positive ionization mode

| Analyte | MW (g/mol) | Retention time (min) | Precursor ion (m/z) | Product ions (m/z) | CE (eV) |
|---|---|---|---|---|---|
| Imidacloprid | 255.66 | 6.86±0.09 | 256.2 | 175,2[a] | 16 |
| | | | | 209,1 | 14 |
| Thiamethoxam | 291.72 | 5.87±0.13 | 292.2 | 132,0 | 18 |
| | | | | 211,2[a] | 13 |
| Thiacloprid | 252.72 | 7.68±0.07 | 253.2 | 126,1[a] | 18 |
| | | | | 186,1 | 12 |
| Clothianidin | 249.68 | 6.78±0.08 | 250.2 | 132,1 | 14 |
| | | | | 169,2[a] | 10 |
| Acetamiprid | 222.68 | 7.16±0.11 | 223.2 | 56,3 | 15 |
| | | | | 126,1[a] | 18 |

[a]Quantification ion

The extraction and clean-up procedure was carried out following the method described by Seccia et al. (2008) with some modifications. Freeze-dried milk (0.5 g) was transferred in test tubes and solubilized with water (4.8 mL) to obtain a final volume of 5 mL. The test tubes were mixed on a vortex shaker for 10 seconds and the reconstituted milk was loaded onto a dry ChemElut. After the liquid has drained into the cartridge under gravity, it was necessary to wait for 15 minutes in order to obtain an even distribution on the filling material. The cartridge was then eluted with three 5 mL portions of dichloromethane. The eluate was collected in a glass centrifuge tube and reduced to dryness using a Univapo (Martinsried, Germany). The dry residue was then reconstituted with 1.0 mL of methanol, briefly sonicated and mixed on a vortex shaker for 10 seconds. The samples were finally centrifuged at 15000xg for 15 seconds in Eppendorf microtest tubes before being transferred to glass vials for LC–MS/MS analysis.

For the validation of the analytical method for LC-MS/MS detection of five neonicotinoids in cow and sheep milk samples, the parameters considered were linearity and range, selectivity, repeatability, reproducibility, recovery, limit of detection (LOD), and limit of quantification (LOQ). The evaluation of linearity and range was assessed in matrix-matched calibration curves, which included 6 concentration levels in the range 1-100 µg/kg. Calibration standards were prepared by fortifying blank milk samples with multicomponent solutions containing the 5 neonicotinoids at appropriate concentration levels. Matrix matched calibration was used to compensate for potential matrix effects occurring during sample preparation and analysis. Selectivity and matrix effects were checked by analysis of blank samples and spiked samples to verify the possible presence of interfering substances at the retention times of the compounds of interest. The repeatability was calculated as the relative standard deviation (RSD %) of results obtained after fortifying 6 blank samples at 3 concentration levels (1, 5, 10 µg/kg) for a total of 18 determinations. The spiked samples were prepared and analyzed with the same instruments, on the same day, and by the same operators. For the evaluation of the within-laboratory reproducibility, 6 blank samples fortified at 3 concentration levels (1, 5, 10 µg/kg) were prepared and analyzed under the same chromatographic conditions on 3 different days by different operators (54 determinations in total), and the relative standard deviation (RSD %) of the replicate measurements was calculated. The recovery rate was evaluated at 3 concentration levels (1, 5, 10 µg/kg) in 6 replicates and on 3 different days by comparing the mean measured concentration in spiked samples with pure standard solutions at the same concentration levels. The limit of quantification (LOQ) was determined as the lowest concentration of analyte in sample, which has

been demonstrated to be accurately quantified by the method. The limit of detection (LOD) was estimated as the concentration level resulting in a peak area of 3 times the baseline noise measured in blank milk extracts at the retention times of the analytes.

## Results and Discussion

The calibration curves generated for the five neonicotinoids were linear and reflect a high correlation between analytical signal and concentration of the analytes. In fact, the coefficients of determination ($R^2$) were always >0.999 over the range LOQ-100 µg/kg. The selectivity of the method was satisfactory with no interfering peaks from endogenous compounds at the retention time of the tested pesticides in milk samples. The analytical results also showed that the matrix effect is negligible in determining the target compounds.

Possible carry-over effects were tested with blank injections after analysis of spiked blank milk samples, but no effects were observed. Average retention times for acetamiprid, imidacloprid, clothianidin, thiacloprid, and thiamethoxam were 7.16±0.11, 6.86±0.09, 6.78±0.08, 7.68±0.07, and 5.87±0.13 minutes, respectively. The repeatability and reproducibility tests, based on intraday and interday measurements, showed relative standard deviations (RSDs) ranging from 4.3% to 31.2% and from 11.6% to 30.0%, respectively (Table 2).

Recovery was checked at 3 spike levels, and the average recovery percentages were found to be between 75.1% and 88.3%. The data about the percent recoveries and the mean recoveries for each fortification level of the 5 pesticides are shown in Table 3. The LOQ and the LOD were the same for all the 5 neonicotinoids, and were calculated to be 0.5 and 1 µg/kg, respectively. Finally, in this study 68 milk samples were analysed and none of the five neonicotinoid pesticides was found above the LOD.

**Table 2** Results of repeatability and reproducibility tests (expressed as RSD%) calculated for each neonicotinoid at 3 spiked levels

| Analyte | Spiking level (µg/kg) | Repeatability | | | Reproducibility | | |
|---|---|---|---|---|---|---|---|
| | | Mean (µg/kg) | SD[a] (µg/kg) | RSD[b] (%) | Mean (µg/kg) | SD[a] (µg/kg) | RSD[b] (%) |
| Imidacloprid | 1 | 0,8 | 0.10 | 12.5 | 0,8 | 0.19 | 23.8 |
| | 5 | 4.2 | 0.33 | 7.8 | 4.5 | 0.68 | 15.1 |
| | 10 | 8.3 | 2.40 | 28.8 | 7.8 | 1.57 | 20.1 |
| Thiamethoxam | 1 | 1.3 | 0.12 | 9.1 | 1.0 | 0.25 | 24.0 |
| | 5 | 4.0 | 0.44 | 11.0 | 4.2 | 1.28 | 30.0 |
| | 10 | 8.1 | 2.52 | 31.2 | 7.4 | 1.88 | 25.1 |
| Thiacloprid | 1 | 0.9 | 0.04 | 4.3 | 0.9 | 0.10 | 11.6 |
| | 5 | 3.2 | 0.48 | 15.1 | 4.3 | 1.00 | 23.1 |
| | 10 | 6.3 | 1.39 | 22.1 | 7.4 | 1.70 | 22.9 |
| Clothianidin | 1 | 0.7 | 0.19 | 25.5 | 0.8 | 0.19 | 24.7 |
| | 5 | 3.4 | 0.66 | 19.6 | 4.1 | 0.94 | 23.1 |
| | 10 | 5.5 | 0.43 | 7.9 | 6.7 | 1.11 | 16.7 |
| Acetamiprid | 1 | 0.8 | 0.11 | 13.5 | 0.8 | 0.17 | 20.5 |
| | 5 | 3.6 | 0.45 | 12.5 | 4.1 | 0.74 | 17.6 |
| | 10 | 7.1 | 1.37 | 19.3 | 7.4 | 1.49 | 20.1 |

[a] Standard deviation
[b] Relative SD

**Table 3** Recovery data of the method for analysis of neonicotinoids in blank milk samples spiked with 3 concentration levels for each analyte

| Analyte | Recovery (%)[a] | | | |
|---|---|---|---|---|
| | Spiking level (µg/kg) | | | M[b] |
| | 1 | 5 | 10 | |
| Imidacloprid | 81.6 | 90.1 | 78.3 | 83.3 |
| Thiamethoxam | 105.3 | 85.0 | 74.8 | 88.4 |
| Thiacloprid | 87.8 | 86.4 | 74.5 | 82.9 |
| Clothianidin | 77.5 | 81.3 | 66.6 | 75.1 |
| Acetamiprid | 80.3 | 83.3 | 74.0 | 79.2 |

[a] Average of 18 replicates at 3 concentrations
[b] Average recoveries of the 3 spiking levels

In pesticide residues analysis, satisfactory values for LOQ should be below or equal to Maximum Residues Levels (MRLs) (SANTE, 2017). Based on this criterion, we can consider that the analytical method adopted in this study showed good sensitivity since the lowest Codex MRL for these neonicotinoids in milks is 0.05 mg/kg (Codex Pesticides Residues in Food Online Database), as shown in Table 4.

**Table 4** Codex Maximum Residue Levels (MRLs) for imidacloprid, thiamethoxam thiacloprid, clothianidin, and acetamiprid in milks

| Neonicotinoid | MRLs in milks (mg/kg) |
|---|---|
| Imidacloprid | 0.10 |
| Thiamethoxam | 0.05 |
| Thiacloprid | 0.05 |
| Clothianidin | 0.20 |
| Acetamiprid | 0.20 |

Despite the importance of milk as a food source, only few studies have reported on the analysis of neonicotinoids (and other pesticides) in this food. Dagnac et al. (2009) developed an original multi-residue method based on dispersive solid-phase extraction (DSPE) with octadecyl (C18) DSPE sorbents followed by LC-MS/MS analysis for the determination of 44 pesticides, including imidacloprid, in milk. LOQ and LOD for imidacloprid were 0.199 and 0.06 µg/L, respectively. An analytical method for quantification of 7 neonicotinoids in different foods was reported by Liu et al (2010). The protocol included acetonitrile extraction, cleanup procedures using SPE HLB cartridges and analysis by UPLC-MS/MS. The LOQs of the neonicotinoids in milk ranged from 0.37 to 2.0 µg/kg. Tian (2011) reports an analytical method for the determination of chloramphenicol, enrofloxacin and 29 pesticides residues, including imidacloprid, in milk. Residues of the targets were extracted with acetonitrile, cleaned up by C18-SPE cartridge, and then determined by HPLC–MS/MS. LOQ and LOD for imidacloprid were 1.9 and 0.6 µg/kg, respectively. A very sensitive UHPLC−MS/MS method for the quantitation of 5 neonicotinoids in milk was developed by Lachat and Glauser (2018). Using liquid−liquid extraction (LLE) starting from 0.5 mL of milk, lowest limits of quantitation (LLOQ) equal or lower than 10 ng/L for all analytes were achieved. In the analytical procedure applied in the present study, compared with those cited above, extraction and purification of the neonicotinoids is made in only one step by loading diluted milk directly onto Chem Elut SPE

cartridges. The process is thus rather simple and fast, while in terms of analytical performance, the method applied, while not being the most sensitive, is satisfactory in consideration of the MRL for milk.

The results of the present survey show that the milk analyzed is safe for human consumption, at least as regards the presence the five neonicotinoids which have been investigated. Milk and dairy products, which constitute essential components in the diet of many communities in Jordan, are a possible source of human exposure to pesticides (Salem et al. 2009; Sait et al. 2010). Several studies have been carried out in Jordan to monitor specific classes of pesticides in agricultural products and in food of animal origin. The presence of Organochlorine pesticides (OCPs) in animal products was reported by Ahmad et al. (2010), Antary et al. (2012), and Alawi et al. (2016). Other studies have also documented their presence in mother milk (Nasir et al. 1998; Al Antary et al. 2015). Jordan has banned the use of OCPs in 1980 and these compounds have been replaced with relatively safe pesticides from different groups, mainly organophosphates, carbamates, pyrethroids, insect growth regulators, and neonicotinoids (Alawi et al. 2012). However, the above-mentioned surveys have shown that although most of OCPs are no more used in Jordan, they can still be detected as residues in agricultural and animal products, probably as a result of environmental contamination. Batarseh and Tarawneh (2013) assessed the presence of 16 pesticides (organochlorine, organophosphate, triazol, pyrethroid and dicarboximide pesticides) in 40 soil samples collected from the southern Jordan Valley region during the period 2011-2012, showing that undesirable amounts of certain pesticides can still be found in environmental media. A recent survey focuses on the determination of three pesticides (abamectin, imidacloprid, and ß-cyfluthrin) in groundwater samples collected from water boreholes of the shallow aquifer systems in Jericho and Jeftlik areas, lower Jordan Valley. Imidacloprid was the pesticide detected most frequently and in the highest concentration. In fact, of the 25 wells sampled, it was found in 24 wells in concentrations ranging between 1.6 and 325.0 μg/kg. This study shows that these pesticides, and in particular imidacloprid, have been used heavily and in an improper way, increasing the risk of adverse environmental and public health effects (Marei et al. 2017).

In conclusion, in the present study the analytical performance results proved that the method adopted was fit for the quantitative determination of five neonicotinoids in milk samples, and generated reliable results. The absence of measurable residues in the analyzed samples might be due to fact that these pesticides were used properly, not in a massive way and/or not during the grazing period. Of course, this assumption should be confirmed by further surveys conducted on other milk samples collected in different periods of the year.

**Conflict of interest** The authors declare that they have no conflict of interest.

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