Simultaneous Analysis of Seven Neonicotinoids in Commercial Milk Samples Using an UHPLC-MS/MS Method

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Abstract: A liquid–liquid extraction and ultra high-performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method was developed for simultaneous analysis of the residues of seven neonicotinoid insecticides (NEOs), namely acetamiprid, clothianidin, dinotefuran, imidacloprid, thiacloprid, thiamethoxam, and nitenpyram, in commercial milk samples. The method had a good linearity (R^2 > 0.992) and a limit of detection range of 0.004–0.15 µg/kg. The average recovery range was 89–119% with an intraday precision of 1.4–10.3% at spiking levels of 8, 12, and 16 µg/kg. The validated method was employed for routine analysis of the aforementioned seven NEOs in commercial milk samples obtained randomly from a supermarket in Miaoli (Taiwan). The detected thiamethoxam and clothianidin levels were 3.4 and 80 µg/kg, respectively.

Keywords: neonicotinoid; UHPLC-MS/MS; commercial milk

1. Introduction

Since the launch of neonicotinoids (NEOs) into the market in the early 1990s to replace organophosphate, carbamate, and pyrethroid insecticides, they have become the most widely used insecticides worldwide. NEOs, including acetamiprid (ACE), clothianidin (CLO), dinotefuran (DIN), imidacloprid (IMI), thiacloprid (THI), thiamethoxam (THIA), and nitenpyram (NIT), are systemic insecticides. Globally, NEO use has continued to increase, with the total production valued at US$ 2.5 billion and with registrations in >120 countries [1]. NEOs are highly water soluble, and because of their systemic mode of action, they have been detected in soils, plants, surface water, pollens, honey, and food [2–6]. Therefore, they may be hazardous to ecosystems and human health through different routes, including ingestion (e.g., contaminated food, water), inhalation, and dermal contact. Toxicological data from animal studies indicate possible genotoxicity, cytotoxicity, impaired immune function, and reduced growth at low levels [7]. The European Food Safety Authority (EFSA) Panel on Plant Protection Products and their Residues stated that ACE and IMI may damage the developing human nervous system [8].

Milk is an indispensable nutritional food in the daily diet of humans. To ensure the safety of milk for consumers, governmental agencies have established maximum residue limits (MRLs) for some NEOs. For NEO residues in milk, the Codex Alimentarius Commission has set the MRL range of 20–200 µg/kg [9]. To accurately determine NEO residues in milk, a fast, sensitive, and robust analytical procedure is vital. From the analytical perspective, milk is a challenging matrix because of its high content of fats, which are extracted along with analytes.
Several methods have been proposed for determining the levels of NEO residues in food samples, including honey, fruits and vegetables, rice, tea, chicken, pork, and eggs [4,10–12]. For solid samples, the most widely proposed pretreatment method is the so-called quick, easy, cheap, effective, rugged, and safe (QuEChERS) technique, followed by solid-liquid extraction. For liquid samples, solid-phase extraction (SPE), liquid–liquid extraction, and dispersive liquid–liquid microextraction are common [12]. Little information is available regarding the quantitative analysis of NEO residues in milk. Some pretreatment methods have been proposed for the analysis of NEOs in milk, such as SPE with the Chem Elut cartridge [13,14], hydropilic–lipophilic-balanced (HLB) cartridge [10], C18 cartridge [15,16], liquid–liquid extraction [17], ionic liquid-magnetic nanocellulose microextraction [18], and the QuEChERS method [19,20].

With regard to the analytical methods, NEOs quantification in milk are mainly determined by high-performance liquid chromatography (HPLC) coupled to diode-array detection (HPLC–DAD) [14], HPLC with DAD and a mass spectrometry (HPLC–DAD/MS) [18], and HPLC or ultra HPLC (UHPLC) coupled to tandem mass spectrometry [(U)HPLC–MS/MS] [10,13,15–19]. However, some methods have limitations in terms of instrument sensitivity and the number of NEOs that can be simultaneously analyzed. In order to achieve very accurate quantification and very low detection limits, the use of stable isotopically labeled standards (IS) UHPLC–MS/MS are essential.

In this study, a method was developed for simultaneously determining the levels of seven NEOs (ACE, CLO, DIN, IMI, THI, THIA, and NIT) residues in milk, and it was validated for the analysis of commercial milk samples in Taiwan. The proposed method is fast, accurate, and sensitive, and it is a liquid–liquid extraction and IS UHPLC−MS/MS method.

2. Materials and Methods

2.1. Reagents and Calibrators Preparation

The analytical standards of six NEOs (≥99.5%), namely ACE, CLO, IMI, THI, THIA, and NIT, were purchased from Sigma-Aldrich (St. Louis, MO, USA). DIN was obtained from ChemService (West Chester, PA, USA). Thiamethoxam-d₃, clothianidin-d₃, imidacloprid-d₄, and acetamiprid-d₃ internal standards (IS) were obtained from Sigma-Aldrich. Analytical-grade acetic acid (99.7%) and HPLC-grade acetonitrile (ACN) and ethyl acetate (EA) were acquired from J. T. Baker (Phillipsburg, PA, USA). Ultra-pure water was obtained using the Milli-Q system (Millipore, Burlington, MA, USA). A total of 31 commercial milk samples were randomly purchased from supermarkets in Miaoli (Taiwan), and were stored at 4 °C. The standard stock solutions of each NEO analyte (1000 mg/L) were prepared in ACN and stored at −20 °C. The multicomponent standard working solutions (10 mg/L) were prepared by diluting each standard solution with ACN and were used for spiking the samples and preparing calibration curves of solvents and the milk matrix. Solutions of five concentrations (0.5, 1, 10, 25, and 50 µg/L) were obtained by diluting standard solutions with ACN for preparing calibration curves of the seven NEOs. A blank milk sample was previously analyzed using UHPLC–MS/MS that the sample did not contain any target analyte. Matrix-matched calibration standards, prepared by spiking a blank sample extract, were in the 0.2–20 µg/kg range.

2.2. NEOs Extraction

Liquid–liquid extraction was performed using the following method described by Lachat and Glauser with minor modifications [17]. The milk samples (0.5 g) were spiked with IS (100 µL, 40 pg/µL in ACN) and 10% acetic acid (20 µL) and were placed in 2-mL microcentrifuge tubes. These tubes were vortexed for 30 s and centrifuged at 14,000 g for 15 min. Supernatants were reserved, and 0.25 mL of H₂O was added to tubes containing pellets. The tubes were shaken in a mixer (Getech, Taipei, Taiwan) for 30 min and subsequently recentrifuged for 5 min. Supernatants from both centrifugations were combined in 2-mL tubes and extracted twice with 0.9 mL of EA. Upper phases were transferred to a
new 2-mL tube and evaporated to dryness under a gentle nitrogen stream. Residues were reconstituted in 200 μL of 50% ACN, and solutions were vortexed and filtered through 0.22-μm PTFE filters.

2.3. UHPLC-MS/MS Analysis

NEOs were analyzed using UHPLC–MS/MS (8045, Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometry instrument with an electrospray ionization source. The analytical column used was a Syncronis C8 column (50 mm × 2.1 mm, 1.7 μm, Thermo) at a flow rate of 0.2 mL/min. Mobile phase A was H2O, and mobile phase B was ACN. The applied elution gradient was as follows: 1% B in 0.5 min, 1–65% B in 4.5 min, and 65–100% B in 2 min. Subsequently, the system was maintained at 100% B for 2 min and re-equilibrated at 1% B for 5.5 min. The total run time and injection volume was 14.5 min and 10 μL, respectively. Ions were monitored in the positive multiple reaction monitoring (MRM) mode. Table 1 and Figure A1 present MS parameters and ion transitions for quantification and qualification of each compound. Figure 1 presents the chromatograms for a spiked milk sample (16 μg/kg of each analyte and 8 μg/kg of IS).

| Analytes       | RT (Min) | Transition 1 (Quantifier) | CE 1(V) | Transition 2 (Qualifier) | CE 2(V) |
|----------------|----------|----------------------------|---------|--------------------------|---------|
| Dinotefuran    | 4.74     | 203→129.05                 | 13      | 203→113.10               | 10      |
|                |          |                            |         | 203→87.10                |         |
| Nitenpyram     | 5.34     | 271→225.00                 | 20      | 271→189.00               | 21      |
|                |          |                            |         | 271→99.00                |         |
| Thiamethoxam   | 5.96     | 292→211.05                 | 14      | 292→181.05               | 24      |
| d3-Thiamethoxam| 5.93     | 295→214.10                 | 12      | 295→132.00               | 25      |
| Clothianidin   | 6.34     | 250→169.10                 | 12      | 250→132.00               | 21      |
| d3-Clothianidin| 6.26     | 253→172.10                 | 30      | 253→132.00               | 33      |
| Imidacloprid   | 6.43     | 256→209.05                 | 16      | 256→175.05               | 18      |
| d4-Imidacloprid| 6.45     | 260→213.05                 | 14      | 260→179.10               | 21      |
| Acetamiprid    | 6.66     | 223→126.05                 | 18      | 223→156.10               | 42      |
| d3-Acetamiprid | 6.61     | 226→126.05                 | 19      | 226→159.00               | 16      |
| Thiacloprid    | 7.16     | 253→126.00                 | 22      | 253→90.00                | 45      |

RT: Retention time; CE: Collision energy.

![Figure 1](Cont.)
Figure 1. Cont.
Figure 1. Representative chromatograms of a milk sample spiked seven neonicotinoids standards at 16 μg/kg and 8 μg/kg of IS.
3. Results and Discussion

3.1. Methodological Evaluation

The analytical method was validated for linearity, limit of detection (LOD), limit of quantification (LOQ), matrix effect, precision, and accuracy. Thiamethoxam-\text{d}_3, clothianidin-\text{d}_3, imidacloprid-\text{d}_4, and acetamiprid-\text{d}_4 were used as IS for THIA, CLO, IMI, and ACE, respectively. In order to improve accuracy and precision, thiamethoxam-\text{d}_3 was used as IS for normalizing the response of the analytes lacking their specific IS. Linearity was evaluated using solvent and matrix-matched calibration standards. The matrix effect was evaluated by comparing standards in solvent with matrix-matched calibration standards. Calibration curves of solvents and the milk matrix were obtained by plotting the quotients of peak areas of THIA, CLO, IMI, and ACE and their corresponding \text{d}_4-labeled IS versus the concentrations of the standards. The proposed method exhibited a good linearity of $>0.992$ in the range of 0.2–20 µg/kg with linear correlation coefficients $R^2$ (Table 2). The matrix effect of the seven NEOs was between 69% and 109%, indicating that the matrix effect is negligible for NEOs, except for THIA. Consequently, the matrix-matched standard solutions were selected for calibration. LOD and LOQ were defined as the level with a signal-to-noise ratio of 3 and 10, respectively, and were in the range of 0.004–0.15 and 0.01–0.36 µg/kg, respectively.

**Table 2.** Matrix effect (ME), limit of detection (LOD), and limit of quantification (LOQ) of seven neonicotinoids.

| Analytes           | Matrix-Matched Calibration Curve in Milk | $R^2$ | Calibration Curve in Solvent | $R^2$ | Matrix Effect (%) | LOD (µg/kg) | LOQ (µg/kg) |
|--------------------|-----------------------------------------|-------|-------------------------------|-------|-------------------|-------------|-------------|
| Dinotefuran        | $y = 28910x + 258143$                   | 0.996 | $y = 31341x - 17851$          | 0.996 | 92                | 0.13        | 0.36        |
| Nitropyrin         | $y = 4070.3x - 9237.4$                  | 0.999 | $y = 3733.6x - 2563.6$        | 0.992 | 109               | 0.05        | 0.17        |
| Thiamethoxam       | $y = 0.1197x + 0.8938$                  | 0.999 | $y = 0.1733x + 0.3865$        | 0.992 | 69                | 0.01        | 0.03        |
| Clothianidin       | $y = 1.4532x - 2.296$                   | 0.999 | $y = 1.6925x - 3.7207$        | 0.999 | 86                | 0.01        | 0.03        |
| Imidacloprid       | $y = 0.1361x - 0.0283$                  | 0.999 | $y = 0.1445x + 0.2521$        | 0.994 | 94                | 0.02        | 0.05        |
| Acetamiprid        | $y = 0.0121x - 0.0014$                  | 0.999 | $y = 0.012x - 0.001$          | 0.999 | 101               | 0.06        | 0.20        |
| Thiacloprid        | $y = 602482x + 304377$                  | 0.999 | $y = 620973x + 329412$        | 0.999 | 97                | 0.004       | 0.01        |

The precision and accuracy of the method were evaluated using blank milk samples spiked with three levels (8, 12, and 16 µg/kg). Intra- and inter-day precisions were determined using the relative standard deviation (RSD). Intraday precision was analyzed by comparing standard deviation in the recovery percentage of the spiked samples run on the same day. Interday precision was determined by analyzing the spiked samples over three different days. Excellent recoveries were obtained at the three spiked levels, and the average recovery range was 89–119% with intra- and inter-day RSD ranges of 1.3–10.3% and 2.1–23.8%, respectively (Table 3). Thiamethoxam-\text{d}_3 was applied for normalizing the response of the three analytes lacking their specific IS, which improve accuracy and precision successfully. Further studies with the isotope labeled standards are essential and encouraged for very accurate quantification in order to compensate matrix effects and mitigate measurement uncertainty [21]. The method provided satisfactory results for detecting trace NEOs in the milk samples because the lowest MRL in milk for these NEOs was 20 µg/kg.

**Table 3.** Total recoveries and intra- and inter-day relative standard deviation (RSD) of seven neonicotinoids ($n = 9$ replicates).

| Analytes   | Precision (% RSD, $n = 9$) | Accuracy (% , $n = 9$) |
|------------|----------------------------|-------------------------|
|        | Intra-Day | Inter-Day |        | Intra-Day | Inter-Day |        | Intra-Day | Inter-Day |        |
|          | 8 µg/kg  | 12 µg/kg | 16 µg/kg | 8 µg/kg  | 12 µg/kg | 16 µg/kg | 8 µg/kg  | 12 µg/kg | 16 µg/kg |
| Dinotefuran | 9.6      | 2.0      | 2.0      | 15.8     | 7.1      | 4.3      | 101      | 113      | 97      |
| Nitropyrin  | 10.3     | 1.4      | 4.1      | 23.8     | 2.1      | 19.5     | 89       | 119      | 104     |
| Thiamethoxam | 8.1      | 4.4      | 2.8      | 13.3     | 14.1     | 10.8     | 102      | 107      | 102     |
| Clothianidin | 3.7      | 8.1      | 7.5      | 12.2     | 10.1     | 11.0     | 103      | 103      | 105     |
| Imidacloprid | 9.9      | 5.6      | 3.2      | 17.8     | 8.0      | 6.7      | 102      | 103      | 101     |
| Acetamiprid  | 2.8      | 1.4      | 2.3      | 8.5      | 5.5      | 8.6      | 109      | 92       | 97      |
| Thiacloprid  | 8.8      | 1.6      | 1.3      | 17.7     | 4.5      | 5.2      | 110      | 100      | 96      |
3.2. Method Application

The method was validated by analyzing 31 commercial milk samples purchased randomly from the supermarket. The results revealed that only THIA and CLO were detected in milk samples. Among the 31 samples, one sample contained THIA (3.4 µg/kg), and one sample contained CLO (80 µg/kg). To the best of our knowledge, this is the first study conducted on NEO levels for a set of the milk samples in Taiwan. The results indicated that NEO residue levels present in milk are safe for human consumption. Lin et al. assessed NEO residues in 128 food samples collected from four parts of Taiwan and reported the highest detection rate for ACE among NEO residues (59%), with the highest level of 80.5 µg/kg [5]. Moreover, at these concentrations, NEO residues in the diet of preschool children in Taiwan did not exceed the hazard index of 1.

Few studies have reported NEOs through milk analysis despite the importance of milk as a food. Adelantado et al., reported an ionic-based ultrasound-assisted dual magnetic nanocellulose microextraction method followed by HPLC–DAD–MS in milk samples [18]. The LOQ range of the six NEOs was 20–60 µg/kg. Seccia et al. developed an SPE technique with Chem Elut cartridges followed by HPLC–DAD for determination of the levels of four NEOs in bovine milk [14]. The LOQ range of the four NEOs was 10–40 µg/kg. Fedrizzi et al. reported a similar pretreatment method that used Chem Elut SPE cartridges, but analysis was conducted through HPLC–MS/MS [13]. The LOQ of five NEOs was 1 µg/kg in sheep and cow milk samples. Liu et al. reported SPE with HLB cartridges followed by UPLC–MS/MS, and the LOQ range of the seven NEOs was 0.37–2.0 µg/kg in milk samples [10]. Kamel et al. and Aguilera-Luiz et al. et al. developed SPE with C18 cartridges followed by UHPLC–MS/MS for analysis of the levels of five and four NEOs in milk samples [15,16]. The LODs of the NEOs ranged from 0.01 and 0.05 µg/kg. Anand et al. reported a QuEChERS method followed by HPLC–MS/MS, and the LOQ range of the four NEOs was 1.0–2.0 µg/kg in human milk [19]. Lachat and Glauser proposed a very sensitive UHPLC–MS/MS method with an LOQ of ≤10 ng/L for determination of the levels of five NEOs in milk [17]. Compared with the aforementioned techniques, this study established a simple, fast, efficient and low cost liquid-liquid extraction–UHPLC–MS/MS method with satisfactory results for MRL in milk. The method developed shows satisfactory validation parameters in linearity, low LOD, accuracy and precision and fits for the simultaneous determination of seven NEOs in milk samples.

The presence of NEOs in breast milk has received increasing interest since human milk is the main nutrition for infants and reveals neonatal exposure. As our knowledge, limited data are available on the NEO residues in breast-milk [17,19]. Exposure of the neonates to NEOs appears to be important, because NEOs are neurotoxicants and may cause neurotoxicity caused by ACE and IMI [8,22]. Future studies are needed to monitor milk levels in breastfeeding women and to study potential risk for NEOs of newborns through milk consumption.

4. Conclusions

In conclusion, this study demonstrated that UHPLC–MS/MS method offers several advantages, including simple preparation, excellent accuracy, and excellent specificity. The proposed simple and fast method is suitable for routine analysis of NEO residues in milk samples and can be helpful for biomonitoring exposure to NEOs in human breast milk.

Author Contributions: Y.-F.H. conceived, designed, analyzed, wrote, and revised the manuscript. H.-J.L. and Y.-M.H. performed the experiments and discussed the results with Y.-F.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.
Appendix A

Figure A1. Cont.
Thiamethoxam; Precursor ion (m/z): 292.0; Collision energy: 14 V.

Figure A1. Cont.
Clothianidin. Precursor ion (m/z): 250.0; Collision energy: 12V.

Figure A1. Cont.
Imidacloprid. Precursor ion (m/z): 256.0; Collision energy: 16 V.

d4-Imidacloprid. Precursor ion (m/z): 260.0; Collision energy: 14 V.

Figure A1. Cont.
Acetamiprid. Precursor ion ($m/z$): 223.0; Collision energy: 18 V.

d$_3$-Acetamiprid. Precursor ion ($m/z$): 226.0; Collision energy: 19 V.

Figure A1. Cont.
Thiacloprid. Precursor ion (m/z): 253.0; Collision energy: 22 V.

**Figure A1.** The respective mass spectra of seven neonicotinoids and isotope labeled standards.

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