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Behavior of Thiamethoxam and Clothianidin in Young Oilseed Rape Plants before Flowering, Monitored by QuEChERS/LC–MS/MS Protocol

Izabela Hrynko [1,*], Gulzhakhan Ilyasova [2], Magdalena Jankowska [1], Ewa Rutkowska [1], Piotr Kaczyński [1] and Bożena Łozowicka [1]

[1] Institute of Plant Protection–National Research Institute, Chelmonskiego 22, 15-195 Bialystok, Poland; m.jankowska@iorpib.poznan.pl (M.J.); e.rutkowska@iorpib.poznan.pl (E.R.); p.kaczynski@iorpib.poznan.pl (P.K.); b.lozowicka@iorpib.poznan.pl (B.Ł.)
[2] Faculty of Natural Sciences, L.N. Gumilyov Eurasian National University, Satpayev 2, Astana 010008, Kazakhstan; iliasova_g@mail.ru
* Correspondence: i.hrynko@iorpib.poznan.pl

Abstract: Nitro-substituted neonicotinoid insecticides have been widely used until recently to control a range of important agricultural pests. Growing concerns about thiamethoxam's toxicity to pollinators have led to its use being restricted or to it even being banned in some countries. Nevertheless, in Asia, Africa, Southeast Europe, and South America thiamethoxam is still used. Although thiamethoxam has been intensively studied all over the world, its dissipation dynamics have not been studied in depth. The subject of the present study was to (1) develop and validate a QuEChERS/LC-MS/MS protocol for the determination of thiamethoxam and its main metabolite clothianidin in samples of young oilseed rape plants with high chlorophyll content, and (2) make a comparison of the degradation behaviors of thiamethoxam and clothianidin in two crops of winter oilseed rape, cultivated on soils with different pH. For determination of thiamethoxam and clothianidin in plant material with high chlorophyll content, a QuEChERS/LC–MS/MS protocol enabling the detection of low levels of compound concentrations was developed. The proposed clean-up protocol provided recoveries within the range of 92–98% for the compounds under analysis. Precision, calculated as relative standard deviation, was below 20%. Satisfactory linearity of the method was obtained in the concentration range under analysis (0.001–1.0 mg kg^{-1}). Differences in degradation of both insecticides, depending on the physico-chemical properties of the soil, were observed. Thiamethoxam and clothianidin residues disappeared in plants very quickly, and they were not detected below the limit of quantitation in oilseed rape at the flowering stage.

Keywords: nitro-substituted neonicotinoids; oilseed rape plants; *Brassica napus*; dissipation; clean-up protocol

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1. Introduction

Brassica napus (oilseed rape, rapeseed) is an important oil crop worldwide [1], with production steadily growing over recent years. In total, 70.62 million tons of oilseed rape were produced worldwide in the season of 2021/2022, which makes this plant the second most predominant oil crop, closely following soya [2,3]. This results from the versatility of the application of rape as a raw material for the production of food, spices, pharmaceuticals, cosmetics, animal feed, and technical fats, including, above all, biofuels. Biofuel from oilseed rape represents 79% of biofuel production in the EU and 13% in the United States. Oilseed rape is also the first valuable forage for honeybees and other insect pollinators during the growing season [4]. Its honey yield, depending on the cultivated variety and the course of weather during the flowering period, is estimated at 80–140 kg ha^{-1}, and the pollen output at 100–150 kg ha^{-1}. However, in the vegetation period, this popular oil

crop is attacked by more than a dozen pest species, which makes insecticide protection extremely difficult and demanding [5,6].

Neonicotinoid insecticides (NIs) were first developed in the early 1990s, and since then the application of this insecticide class has quickly covered a wide range of agricultural products [7,8]. Neonicotinoids may be divided into three groups: N-nitroguanidines (acetamiprid, thiamethoxam, clothianidin, dinotefuran), nitromethylenes (nitenpyram), and N-cyanoamidins (imidacloprid, thiacloprid). Nitro-substituted neonicotinoid insecticides, which include imidacloprid, thiamethoxam (TMX), and clothianidin (CLO), have been widely used until recently to control a range of important agricultural pests, both through foliar applications and also as seed dressings and by soil application [9]. Neonicotinoids can be absorbed by the seeds and roots of plants and translocated to almost all plant organs for managing a wide spectrum of insect pests, which makes them ideal candidates for seed coatings [8].

However, neonicotinoids, like many pesticides, can persist for long periods of time in agricultural soil, resulting in long-term contamination and, in some cases, the accumulation of harmful substances [10–12]. Pollinating insects can be exposed to pesticides not only as a result of the contamination of agricultural crops but also from non-target plants, i.e., weeds and wildflowers classified as melliferous plants [13,14]. In turn, the accumulation of chemicals in plants can lead to harmful substances in honey and other bee products [15,16]. A growing problem in modern agriculture is also the contamination of bee forage with pesticides from spray drift from neighboring fields and the consequent unintentional contamination with pesticides [17,18].

Growing concerns about thiamethoxam's toxicity to pollinators have led to its use being restricted or it even being banned in some countries. Nevertheless, in Asia, Africa, Southeast Europe, and South America thiamethoxam is still used. Seven neonicotinoids account for approximately 17% of the value of the global insecticide market. Confirmation of the use of thiamethoxam is provided by this year's European Commission issued on EU Agri-Food Fraud suspicions "ANUARY 2024 REPORT ON EU AGRI-FOOD FRAUD SUSPICIONS" [19]. It reports that thiamethoxam was detected in rice from India and Pakistan at 0.044 mg kg^{-1} and 0.032 mg kg^{-1}, in ginger syrup from China (0.064 mg kg^{-1}), in passion fruit from Colombia (0.044 mg kg^{-1}), vine leaves from Egypt (0.044 mg kg^{-1}), guava from India (0.20 mg kg^{-1}), or cumin from India (0.14 mg/kg).

Thiamethoxam, (EZ)-3-(2-chloro-1.3-thiazol-5-ylmethyl)-5-methyl-1.3.5-oxadiazinan-4-ylidene(nitro) amine, is effective in killing sucking and chewing insects, such as aphids, whiteflies, lovebugs, thrips, and beetles, attacking rice, oilseed rape, maize, cotton, vegetables, or mango [20]. Thiamethoxam is a crystalline, scentless compound with a melting point of 139 °C. It shows relatively high solubility in water, amounting to 4.1 g L^{-1} at 25 °C, and a low log P partition coefficient of −0.13 at pH 6.8. Thiamethoxam is transformed into clothianidin in plants, soils, and insects [21,22]. Clothianidin was first reported as one of the most prominent metabolites of thiamethoxam in cotton plants by Nauen et al. [21]. Clothianidin, (E)-1-(2-chloro-1.3-thiazol-5-ylmethyl)-3-methyl-2-nitroguanidine), is structurally similar to thiamethoxam, differing from the CH_2OCH_2 grouping. Its solubility in water is low, amounting to 0.34 g L^{-1}, whereas, in comparison with thiamethoxam, it has a higher octanol–water partition coefficient (Log K_{ow}: 0.905). These compounds are characterized by varied persistence in soil, plants, or living organisms.

The persistence of pesticides in soil and plants depends, among other things, on the physico-chemical properties of soil, the climate conditions, and the physico-chemical properties of the compound itself. The fate of pesticides is also affected by the plant species and the stage of cultivation at the moment of application of the agent [23–27]. Many studies have been devoted to the assessment of the dissipation of neonicotinoids in soil [28–31]. To the best of our knowledge, there are relatively few reports concerning the dissipation of both thiamethoxam and clothianidin in crops. Several authors have investigated the dissipation of thiamethoxam in potatoe [32], tomato [33], pomegranate [34], maize [35], cotton [36], mango [37], and apple [38]; however, as we can see, previously published work

has focused on the dissipation of thiamethoxam after foliar application. Information on the presence of pesticide residues in rapeseed plants is very limited, and only a few literature references concern the content of pesticides in oilseed rape [39].

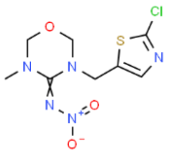
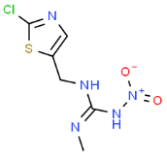
Different approaches to the identification of neonicotinoids and their metabolites in environmental samples (water, soil) [40,41], agricultural samples (cucumber, spinach, apple, pomelo, green pepper, tomato, grain, rice, millet, maize, sugarcane juice, tea, wine) [42–49], and bees and bee products (honey, honey liqueur, beeswax) [50–55] have been proposed. These methods, described in the literature, enable the identification of neonicotinoids in a relatively narrow concentration range not allowing identification of sublethal doses. Since the application of seed dressings contributes to the reduced use of insecticides in spray form and enables the reduction of the quantity of plant protection chemicals released to the environment, there is a need for a thorough risk assessment conducted under real conditions. Therefore, it is necessary to develop analytical methods enabling the detection of low levels of compound concentrations (at a concentration limit of 0.001 mg kg^{-1}). The aim of this study was to (1) develop and validate a QuEChERS/LC–MS/MS protocol (an effective method) for the determination of thiamethoxam and its main metabolite clothianidin in samples of young oilseed rape plants with high chlorophyll content, and (2) make a comparison of the degradation behaviours of thiamethoxam and clothianidin in two crops of winter oilseed rape, cultivated on soils with different pH.

2. Materials and Methods

2.1. Chemicals and Reagents

Thiamethoxam (CAS 153719-23-4) and clothianidin (CAS 210880-92-5) (>98% purity) were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The properties of thiamethoxam and clothianidin are presented in Table 1. Triphenyl phosphate (TPP) as the internal standard (IS) was supplied from Sigma-Aldrich (Sant Louis, MO, USA).

Table 1. Physico-chemical parameters of thiamethoxam (THX) and clothianidin (CLO) determining their translocation capacity within the plant.

Compound	Thiamethoxam (TMX)	Clothianidin (CLO)
Chemical structure		
Molecular formula	$C_8H_{10}ClN_5O_3S$	$C_6H_8ClN_5O_2S$
Molecular weight (g mol^{-1})	291.71	249.7
Melting point (°C)	139.1	176.8
Water solubility (mg L^{-1}) [1]	4100	340
Octanol–water partition coefficient (log K_{ow})	−0.13	0.905
Dissociation constant (pKa) at 25 °C	no dissociation	11.1

[1] Solubility in water at 20 °C at pH 7.

Standard stock solutions of thiamethoxam (1000 mg L^{-1}) and clothianidin (1000 mg L^{-1}) were prepared in methanol. The combined working standard solutions were generated by serial dilution of the stock solution in methanol. The working standard solutions were used for the preparation of matrix-matched standards within the concentration range of 0.001–1.0 µg mL^{-1}. All stock and working standard solutions were protected from direct light and stored in dark glass bottles in a freezer at approximately −4 °C until analysis.

LC-MS grade acetonitrile (ACN), methanol (MeOH), and formic acid (FA) were purchased from Merck (Darmstadt, Germany). Commercial QuEChERS extraction salt packets (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, and 0.5 g disodium citrate sesquihydrate) were purchased from Agilent Technologies (Santa Clara, CA, USA). The QuEChERS sorbent

kits (i) PSA/GCB/$MgSO_4$, (ii) PSA/$MgSO_4$, and (iii) C_{18}/PSA/$MgSO_4$ were supplied by Agilent Technologies (USA) and (iv) $MgSO_4$/PSA/Chlorofiltr/C_{18} was supplied by Sigma-Aldrich (Steinheim, Germany). Ultrapure water (LC grade 18 MΩ cm) was prepared using a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Field Trials and Sampling

Field trials were conducted at two agriculture regions (53°08′45.6″ N 22°45′17.1″ E—Location 1 and 53°01′51.2″ N 23°20′20.2″ E—Location 2) in north-eastern Poland, which produces annually significant amounts of oilseed rape exported to Europe. On two winter oilseed rape fields (with an area of 41 ha—Location 1, and 35 ha—Location 2) were treated with thiamethoxam as a seed dressing, respectively. Winter rape seeds were treated with a commercial preparation of Cruiser 70 WS (thiamethoxam 700 g kg^{-1}; at a rate of 450 g a.s./100 kg seed), in the dose recommended by the manufacturer. An amount of 4 kg of rape seeds were sown per 1 ha to a depth of 2.5–3.5 cm in locations 1 and 2, respectively. The characteristic properties of soil used in the field, such as pH values, were 4.9, and 6.3 for locations 1 and 2, respectively. In addition, the organic matter contents of the soil were 1.7% and 1.6% for locations 1 and 2, respectively (Table 2).

Table 2. The selected physico-chemical properties of the soils from two locations.

Parameter	Location 1	Location 2
pH	4.9	6.3
organic matter parameters	1.7	1.6
granulometric composition	clay < 0.002 mm—4.46%; silt 0.002–0.02 mm—17.62%; sand 0.02–0.05 mm—5.17%; clay 0.05–2.00 mm—72.75%	clay < 0.002 mm—6.05%; silt 0.002–0.02 mm—24.6%; sand 0.02–0.05 mm—8.24%; clay 0.05–2.0 mm—61.12%

Representative samples of the plant material (young oilseed rape plants) were collected when the oilseed rape plants were at the stage of 4–6 leaves unfolded (BBCH 14–16). Subsequent samples were collected at the following time intervals: 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 days. Each sample was taken from 5 different points and weighed approximately 500 g. Untreated samples were collected to be used as a blank. Both dissipation kinetics and residue determination experiments were carried out by the Guideline on Pesticide Residue Trials established by the Ministry of Agriculture and Rural Development of Poland.

2.3. Sample Preparation–Sample of High Chlorophyll Content

The plant material samples were transferred frozen (on dry ice) to the Laboratory of Food and Feed Safety of the Institute of Plant Protection—National Research Institute of Poland, where the analysis was performed. The analytical sample of the plant material was ground in a homogenizer and then mixed to ensure representativeness. All collected samples were stored in a freezer at −20 °C until analysis.

For the determination of thiamethoxam and clothianidin in plant material with high chlorophyll content, a PSA/GCB/$MgSO_4$ clean-up protocol was developed. Ten grams of analytical sample were weighted into 50 mL polypropylene tubes, and 50 µL TPP (triphenyl phosphate) at a concentration 5 µg mL^{-1} (internal standard (IS)) was added. To this, 10 mL of 1% formic acid in acetonitrile was added and the tubes were shaken for 1 min. After that, QuEChERS extraction salts were added to the sample tube to separate the phases; it was then shaken and centrifuged for 5 min at 4500 rpm. Due to high chlorophyll content, sample purification was necessary. To this end, 8 mL of extract was transferred into a 15 mL centrifuge tube containing PSA/GCB/$MgSO_4$. The d-SPE tube was shaken vigorously for 1 min and then centrifuged at 4500 rpm for 10 min. The eluent then was filtered through a 0.45 µm membrane and analyzed using LC–MS/MS (Eksigent Technologies, Dublin, CA, USA). A general scheme of the procedure under consideration is shown in Figure 1.

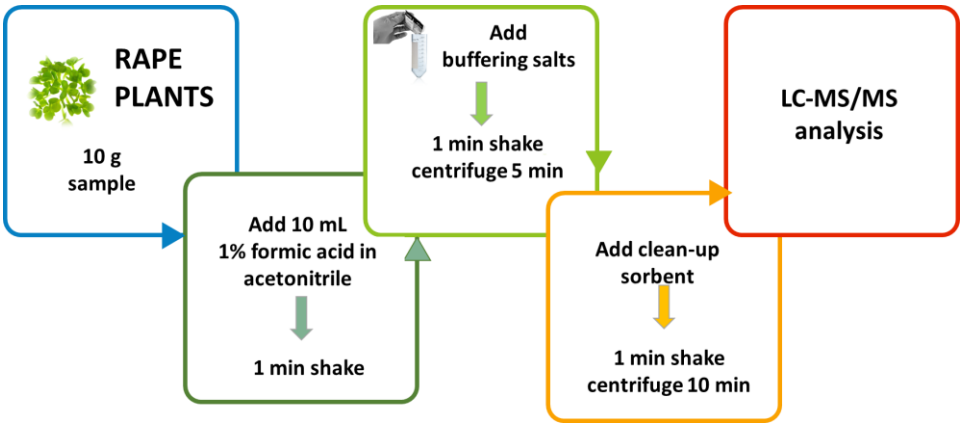


Figure 1. Scheme of sample preparation.

2.4. Method Validation

The developed procedure for the determination of thiamethoxam and clothianidin residues in young oilseed rape plants was subject to a validation process by the guidelines of the European Commission included in the [56] SANTE/11312/2021 "Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed".

2.5. Instrumentation Conditions

The LC–MS/MS system consisted of an Eksigent Ultra LC–100 series (Eksigent Technologies, Dublin, CA, USA) equipped with a KINETEX XB 1.7 µm, 2.1 × 50 mm (Phenomenex, Torrance, CA, USA) analytical column. Analyses of compounds were performed by LC–MS/MS in multiple reaction monitoring (MRM) modes using two specific ion transitions for each analyte (m/z 292.0 → 211.0 and 292.0 → 181.0 for TMX, 250.0 → 169.0 and 250.0 → 132.1 for CLO). An MS/MS 6500 QTRAP system (AB Sciex Instruments, Foster City, CA, USA) was equipped with an electrospray ionization source (ESI). The parameters for thiamethoxam and clothianidin are presented in Table 3. For both methods the following settings were used: ion spray voltage, 4500 V; temperature, 450 °C; curtain gas, 35 psi; ion source gas 1 (nebulizer gas), 60 psi; and ion source gas 2 (auxiliary gas), 70 psi. The mobile phase consisted of water with 0.2% formic acid and 5 mM ammonium formate (phase A) and methanol with 0.2% formic acid and 5 mM ammonium formate (phase B) with a flow rate of 0.4 mL min^{-1}. The elution gradient was as follows: 0–0.5 min (A: 95%, B: 5%), 5–7.5 min (A: 5%, B: 95%), 8–10 min (A: 95%, B: 5%). The injection volume was 5 µL.

Table 3. SRM transitions of thiamethoxam and clothianidin.

Compound	Precursorion (m/z)	Quantification				Confirmation			
		MRM Transition (m/z)	DP (V)	CE (V)	CXP (V)	MRM Transition (m/z)	DP (V)	CE (V)	CXP (V)
Clothianidin	250.0	169.0	6	19	10	132.1	6	21	6
Thiamethoxam	292.0	211.0	61	17	12	181.0	61	31	10

3. Results

3.1. Optimization of d-SPE Clean-Up Protocol

Upon centrifugation and pouring of the extract into a 15 mL tube, the supernatant was subject to four different purification stages to optimize the co-extractives removal. The following sorbent combinations were selected for the testing: (i) PSA/$MgSO_4$, (ii) PSA/GCB/$MgSO_4$, (iii) C_{18}/PSA/$MgSO_4$, and (iv) $MgSO_4$/PSA/Chlorofiltr/C_{18}.

All the aforementioned clean-up sorbents were tested in the treatment of spiked samples to evaluate the co-extractives removal, based on the recoveries of the known-

spiking levels (Figures 2 and 3). The proposed d-SPE clean-up protocol with PSA/$MgSO_4$ sorbent showed poor recoveries (<70%) for clothianidin and thiamethoxam. Furthermore, the application of this sorbent caused the obtaining of a matrix effect outside the acceptable range (−36 for CLO). For $MgSO_4$/PSA/Chlorofiltr/C_{18}, low recoveries were observed for clothianidin. Samples purified with C_{18}/PSA/$MgSO_4$ showed poor clothianidin recoveries. According to the results, the application of PSA/GCB/$MgSO_4$ as the sorbent exhibited the highest extraction efficiency for target analytes. Using this combination provided recoveries within an acceptable range of 40–70% for both compounds. Furthermore, in the case of this combination the compounds were characterized by a negligible matrix effect (−20% < ME < 20%). The purification effects of four sorbents (recoveries and matrix effects) were compared and PSA/GCB/$MgSO_4$ was finally selected (Figures 2 and 3).

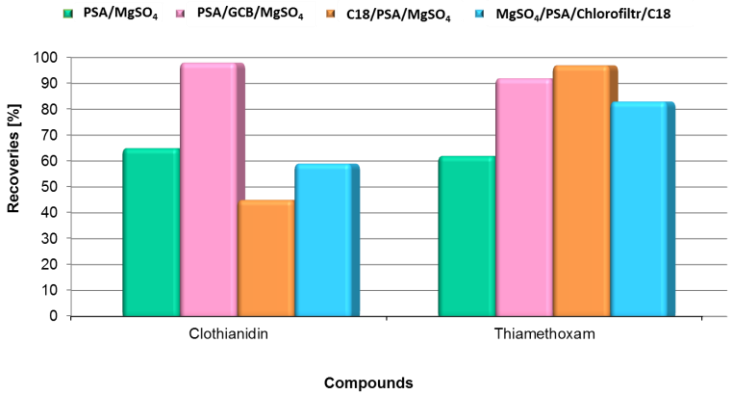


Figure 2. Recoveries of thiamethoxam and clothianidin obtained under different d-SPE clean-up protocols: (i) PSA/$MgSO_4$, (ii) PSA/GCB/$MgSO_4$, (iii) C_{18}/PSA/$MgSO_4$, and (iv) $MgSO_4$/PSA/Chlorofiltr/C_{18}.

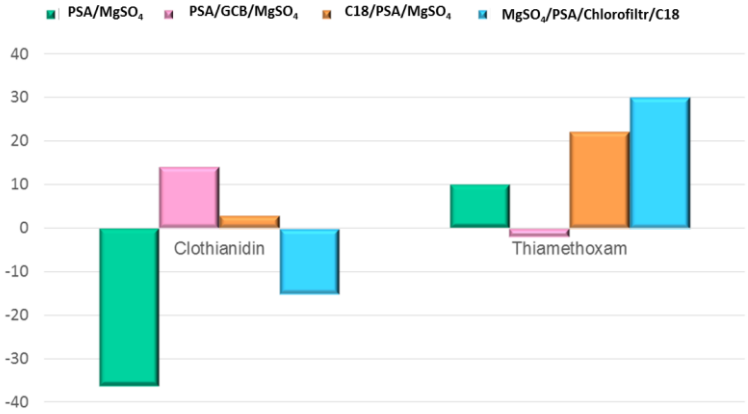


Figure 3. Matrix effects of thiamethoxam and clothianidin were obtained using four different d-SPE clean-up protocols: (i) PSA/$MgSO_4$, (ii) PSA/GCB/$MgSO_4$, (iii) C_{18}/PSA/$MgSO_4$, and (iv) $MgSO_4$/PSA/Chlorofiltr/C_{18}.

The sensitivity and specificity of LC-MS/MS makes it possible to analyse samples of very high complexity and perform analyses of compounds at very low concentration levels. Matrix-matched calibrations were within acceptable quantitation, satisfactory recoveries, and limits of quantitation (LOQs) in rapeseed plants. Thiamethoxam and clothianidin had good linearity in the range of 0.001–1.0 mg kg^{-1}. Average recoveries of thiamethoxam for plants ranged from 92% to 98%, with RSD values of 3.9%–5.2%. The matrix effect for thiamethoxam is 10%, while for clothianidin it is −8%. The developed method could provide reliable residue levels for thiamethoxam. In each series of tests, control samples were analyzed. Samples were fortified with analyzed substances at the limit of quantification. The remaining validation parameters are presented in Table 4. Example chromatograms of

thiamethoxam and clothianidin standards and spiked young oilseed rape plants sample are presented in Figure 4.

Table 4. Validation parameters of the method for determining thiamethoxam and clothianidin residues in young oilseed rape plants by liquid chromatography LC–MS/MS.

Analytes	Thiamethoxam	Clothianidin
R^2	0.99989	0.99988
Matrix effects [1]	10	8
LOQ (mg kg^{-1})	0.001	0.001
Linearity range (mg kg^{-1})	0.001–1.0	0.001–1.0
Recovery (%)	92	98
RSD (%)	5.2	3.9

[1] Matrix effects (ME, %) = [(slope of calibration curves in matrix/slope of calibration on solvent) − 1] × 100%.

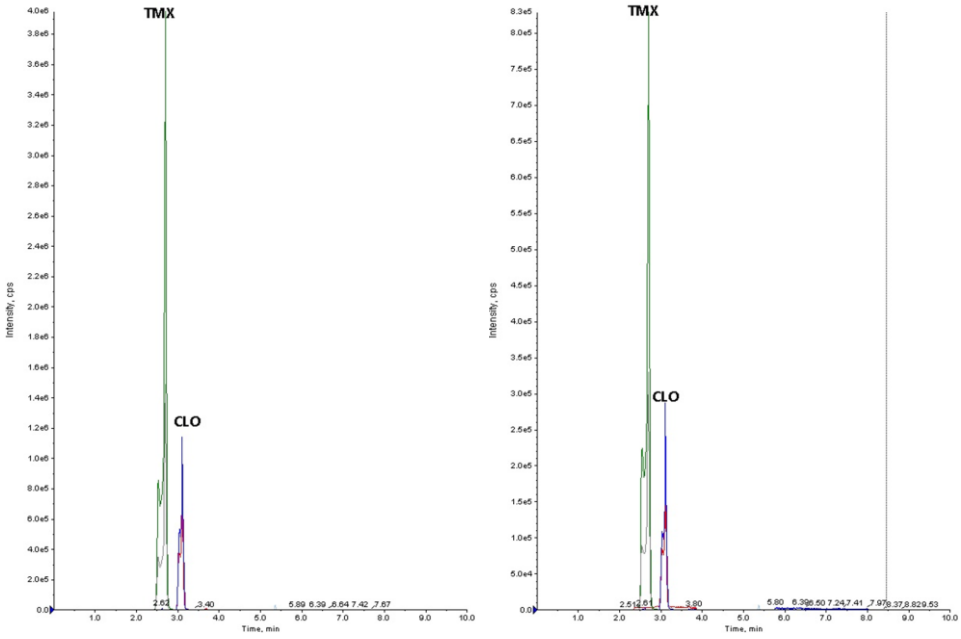


Figure 4. Chromatogram of thiamethoxam and clothianidin standards and spiked blank plants sample.

3.2. Behavior of Thiamethoxam and Clothianidin in Young Oilseed Rape Plants before the Flowering

The residues and dissipation rates of thiamethoxam and clothianidin in oilseed rape plants are summarized in Table 5 and shown in Figure 5. Seeds before sowing were analyzed and the dosage was checked. The initial concentration of thiamethoxam in seeds was 0.315 mg kg^{-1}. For thiamethoxam and its metabolite, two stages may be distinguished in the process of their decay in a plant. The first stage lasted until the 16th day of the experiment, in which the concentration of compounds in a plant was growing, and the second stage started from the 16th day, in which the concentration was declining. Depending on the soil pH, differences in compound concentrations were recorded. Deposits of thiamethoxam and clothianidin were higher in a plant from an acid soil location (Figure 5). In the case of this soil, a gradual but marked increase in thiamethoxam and clothiani-din concentrations was recorded in the plant at 5 initial dates, i.e., day 12, 13, 14, 15, 16. On the other hand, in the case of soil with pH = 6.3, the concentration of thiamethoxam in the plant on these days was more even and remained at a similar level within 0.05 mg kg^{-1}. However, between days 15 and 16, a slight increase in the concentration of clothianidin was observed in the plant.

Table 5. The dissipation (in %) of thiamethoxam (TMX) and clothianidin (CLO) in rapeseed plants cultivated on soils with different pH.

Days after Sawing	TMX	CLO	TMX	CLO
	pH = 4.9		pH = 6.3	
16	-	-	-	-
17	34.6	66.7	40.3	23.0
18	58.2	77.2	66.4	68.3
19	81.3	98.4	76.2	87.0
20	85.6	99.2	77.7	93.2
21	92.4	99.6	90.4	99.4
22	99.2	99.6	98.7	100.0
23	99.6	99.6	99.8	100.0
24	99.9	99.6	99.8	100.0
25	100.0	100.0	100.0	100.0
26	100.0	100.0	100.0	100.0
27	100.0	100.0	100.0	100.0
28	100.0	100.0	100.0	100.0
29	100.0	100.0	100.0	100.0
30	100.0	100.0	100.0	100.0

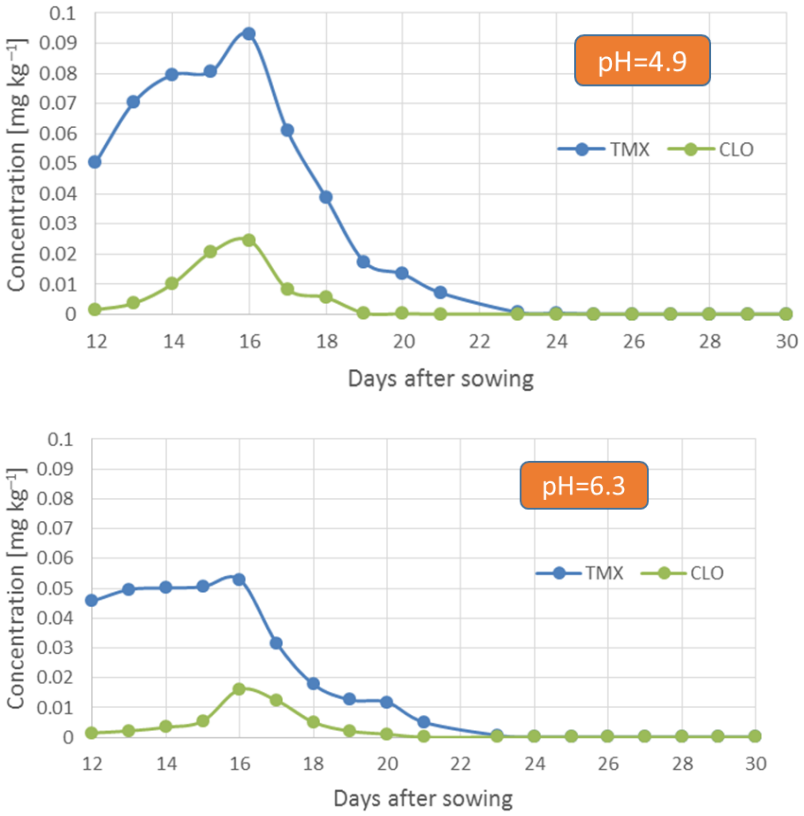


Figure 5. Dissipation pattern of thiamethoxam (TMX) and clothianidin (CLO) in rapeseed plants cultivated on soils with different pH.

Residual thiamethoxam reached a maximum of 0.0929 mg kg^{-1} in acid soil (pH = 4.9) on day 16 and 0.0529 mg kg^{-1} in neutral soil (pH 6.3) on the same day. The following kinetic equation is used for the dissipation of thiamethoxam: $y = 16e^{-0.415x}$, $R^2 = 0.99$ (pH = 4.9); $y = 16e^{-0.422x}$, $R^2 = 0.9894$ (pH = 6.3). The half-life ranged from 1.6 to 1.7 days. Regardless of the soil pH, the concentration of metabolite amounted to 10–20% of the value of thiamethoxam. Residual clothianidin reached a maximum of 0.0246 mg kg^{-1} in acid soil on day 16 and 0.0161 mg kg^{-1} in neutral soil. Clothianidin concentration decreased

according to the exponential equation, $y = 16e^{-0.46x}$, $R^2 = 0.8921$ (pH = 4.9); $y = 16e^{-0.455x}$, $R^2 = 0.9649$ (pH = 6.3). The half-life was 1.5 days for both sites. Thiamethoxam is a polar compound with a low octanol/water partition coefficient (Log K_{ow}: −0.13) (Table 1). If water solubility is higher, translocation through the plant is faster. Accordingly, thiamethoxam residues were detected in samples of crops at higher levels compared to clothianidin (Log K_{ow}: 0.905). Compound residues were monitored until the moment of reaching the limit of quantitation (concentration 0.001 mg kg^{-1}). Depending on the soil pH, differences in the decomposition of thiamethoxam and clothianidin in a young oilseed rape plant were observed (Table 5). For a plant from a site where the soil was acidic on day 17, the percentage of thiamethoxam decomposition was 35%, 58% on day 18, 81% on day 19, and 86% on day 20. In the plant from the second location, the percentage of thiamethoxam decomposition was 40%, 66%, 76%, and 78% on days 17, 18, 19, and 20, respectively. On day 21 for plants from both sites it was above 90%, and on day 24 it reached 99%. The percentages of clothianidin decomposition on the 17th day in a plant grown on acid and alkaline soil were 66.7 and 23.0%, respectively. However, on the 20th day since the seeding, it reached a similar level (above 90%) in both locations. Initially, thiamethoxam was decomposing slightly faster in alkaline soil, whereas quicker decomposition thereof could be observed in acid soil since the 19th day. On the 25th day of the experiment, the degree of decomposition of thiamethoxam and clothianidin was 100%. Alkaline environments accelerate the degradation of pesticides, while acidic soils interfere with the uptake of minerals by plants and risk triggering soluble forms of aluminum [57]. This probably explains the positive correlation between the alkaline pH and the faster rate of thiamethoxam decomposition in the plant in the first few days. Also, the texture and organic matter content of the soil can affect the degradation of thiamethoxam [58]. In our study, the soils at the two locations differed not only in reaction but also in the percentage of each fraction. The acidic soil had the highest proportion of loam fractions (77.21%), with smaller proportions of sand (5.17%) and clay (17.62%). In the alkaline soil, the proportion of the loam fraction was lower (67.15%), while sand (8.24%) and clay (24.6%) were higher.

4. Discussion

4.1. Optimization of d-SPE Clean-Up Protocol

Young oilseed rape plants contain considerable amounts of chlorophyll, which might act as an instrumental interference. Thus, further purification of extraction solutions was needed. Extraction of analytes using the "dispersive SPE (d-SPE) clean-up protocol" facilitates their proper sample clean-up [59]. Therefore, in the present study the d-SPE clean-up protocol was used for the purification of extracts of young oilseed rape plants.

Co-extraction of chlorophyll from green matrices presents a significant difficulty, as it is one of the most problematic interferents in pesticide residue analysis due to its non-volatile properties [60]. In the case of so-called 'green matrices', the sorbent mixture of magnesium sulfate and primary and secondary amine (PSA), commonly used in the purification step, provides unsatisfactory results. Primary secondary amine (PSA) is a base sorbent used for the QuEChERS d-SPE clean-up of fruit and vegetable extracts because it removes many organic acids and sugars [61]. The application of this sorbent to samples containing chlorophyll may result in the matrix interferents contained in the sample not being reduced or removed, which may cause false positive or negative results [62]. Chlorophyll is a problematic component of green matrices and has so far been removed from samples by many researchers using GCB sorbent [63,64]. Graphitized carbon black (GCB) removes colored compounds, such as pigments and chlorophylls. However, this sorbent has a significant drawback, namely, it causes the strong adsorption of polar analytes, resulting in low recoveries [65]. The effectiveness of sorbents such as GCB, PSA, and C_{18} on four different matrices (grain, straw, green plants, soil) was tested in a study by Zhao et al. (2013) [66]. The single sorbent C_{18} they used for green plant samples resulted in satisfactory recoveries within the 70–104% range. In contrast, unacceptable recovery values (below 70%) were obtained for grain, straw, and soil samples. Purification using a single GCB, on

the other hand, gave good results only for grain samples, while the results for the other three matrices were poor. This may be because the target pesticide was selectively retained by the GCB under matrix conditions [67]. The same authors also investigated how the combination of PSA and GCB would affect the removal of chlorophyll and the recoveries obtained. The mixture of these two sorbents proved to be the most effective for green plants. Average recoveries in green plants at the three levels ranged from 76.1 to 100.0%, with relative standard deviations (RSDs) below 10%. These sorbents, individually and in a mixture, were also tested by other researchers using them for the determination of pesticides in green matrices, such as tea leaf samples [68,69]. For example, in a study by Ly et al. (2020) [69] GCB in combination with PSA/$MgSO_4$ proved to be the most effective purification method and allowed the quantification of 225 pesticide residues in green tea leaf samples. Most of the analyzed pesticides were characterized by a non-significant matrix effect ($-20\% < ME < 20\%$).

4.2. Behavior of Thiamethoxam and Clothianidin in Young Oilseed Rape Plants before the Flowering

Although thiamethoxam, a nitro-substituted neonicotinoid, was one of the world's most used insecticides until recently, its dissipation dynamics have not been studied in depth. The dissipation curves reported in the literature are valid only for a given crop under specific conditions [70]. Many factors can contribute to the dissipation of pesticide residues, i.e., the crop (morphology, stage of plant development) or environmental conditions (soil pH) [71]. Different application methods also resulted in different distribution, transfer, and degradation dynamics [72]. Pesticides are usually applied by either foliar spray or soil spray [22]. Hilton et al. (2019) [73] described the dissipation of thiamethoxam in soil under laboratory and field conditions. They noticed that the concentration of clothianidin in experiments with dressed seeds amounted to between 2.1 and 4.5% of the thiamethoxam value. On the other hand, in the case of spraying application, the maximum concentrations of clothianidin fell between 16.3 and 19.0% of the thiamethoxam value. The study by Hilton et al. (2019) [73] examined a number of factors related to the degradation rate of thiamethoxam under field and laboratory conditions. The thiamethoxam spray used was shown to decompose faster in the field than under laboratory conditions. According to the authors, higher temperatures in soil exposed to sunlight in the field may have accelerated the degradation process of thiamethoxam. It was also found that neither the method of application of the product nor the pH of the soil and its organic matter content affected the rate of degradation. Another study by these authors [74] examined the rate of thiamethoxam degradation under field conditions from various locations in Europe. The research conducted showed that thiamethoxam, regardless of the application method used and the prevailing environmental conditions in the fields, degraded <10% of its maximum concentration within a year of application. The levels of clothianidin observed in the study were at very low concentrations, so the authors did not undertake an assessment of the rate of disappearance of this substance in the soil.

However, early work [75] suggested that the half-lives of thiamethoxam were related to factors such as the physico-chemical parameters of soil. Barik et al. (2010) [76] reported that the degradation of thiamethoxam in paddy soil and the half-lives were from 8.4 to 13.1 days. Gupta et al. (2008) [77] studied the degradation of thiamethoxam in soil in India under different moisture conditions and found that the half-life of thiamethoxam varied with moisture content, ranging from 46 to 301 days. In turn, the half-lives of clothianidin ranged from 65 to 35 days. The results in this study indicate that when the moisture content is higher than 20% its effect on the half-lives becomes less significant. Mörtl et al. (2016) [78] studied the mobility of clothianidin and thiamethoxam in three soil types in Hungary. They showed that both insecticides were more mobile in sand than in loam and clay. They reported that the high solubility of thiamethoxam in water may affect the retention of this substance in loam soil. However, they did not conduct research in plants. In another study, El-Aswad et al. (2024) [79] reported that the half-life of thiamethoxam in silty-loam soil

was 15.0 days, in sandy-loam soil 20.1 days, and in loam soil 27.2 days. Thiamethoxam decomposed faster in silty and sandy soil. Ramasubramanian and Paramasivam (2020) [80] studied the dispersal of thiamethoxam in a tropical sugarcane crop ecosystem. They observed that thiamethoxam persists in sandy-loam soil for 60 days and only reaches a level of 0.01 mg kg^{-1} on day 75. The half-life was 16.5 days. In contrast, at twice the recommended dose, the insecticide persisted for up to 75 days, with a half-life of 16.91 days.

Yang et al. (2022) [72] investigated the dissipation behavior of thiamethoxam and its metabolite clothianidin on spinach. Although, like young oilseed rape plants, it is a chlorophyll-rich plant, the degradation of these substances proceeds differently. In the case of spinach, thiamethoxam degraded at a faster rate of 1.3 to 1.6 days in the plant. The results were similar to the half-lives of thiamethoxam in spinach (2.3 days), reported by previous studies [81]. As in our study, thiamethoxam was rapidly degraded in the crop, with clothianidin appearing easily as a plant metabolite. This behavior of the active substances is also confirmed by other researchers [82].

5. Conclusions

The developed QuEChERS protocol with extract clean-up using PSA/GCB/$MgSO_4$, followed by LC–MS/MS analysis, could be used with satisfactory results for the quantification of neonicotinoid insecticide thiamethoxam and its main metabolite clothianidin in green plants, such as oilseed rape. Recoveries of the tested insecticides were between 92% and 98%, and RSDs were consistently < 6%.

Based on the established method, the behavior of two nitro-substituted neonicotinoids thiamethoxam and clothianidin in oilseed rape in two agriculture regions were investigated. The significant effects of soil pH on the recorded concentrations of thiamethoxam and the clothianidin metabolite in the oilseed rape plant were observed. Deposits of thiamethoxam and clothianidin were higher in a plant from an acid soil location (pH = 4.9). Regardless of the soil pH, the concentration of metabolite amounted to 10–20% of the value of thiamethoxam. In both locations, residues of the examined compounds have disappeared in plants very quickly, and they were not detected below the limit of quantitation in oilseed rape at the flowering stage.

The results presented in this paper should be limited to this experiment only and should not be extrapolated to other soil types or climatic conditions. For other types of soil (with different texture, organic matter content, or pH), different results may be obtained. Climatic conditions can affect pesticide dispersal to varying degrees due to changes in plant metabolism, or the bioavailability of pesticides in the soil. Since a similar experiment has not been conducted previously, the present study should be continued under different climate and soil conditions. This will provide data for the risk assessment of the two neonicotinoids.

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