

Development of an Analytical Method of Nereistoxin and Nereistoxin-related Pesticides in Agricultural Products by LC-MS/MS

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Abstract: A simultaneous analytical method was developed for the quantification and confirmation of nereistoxin and nereistoxin-related pesticides in agricultural products. These pesticides were extracted with a 2% L-cysteine solution in 0.05 N HCl and hydrolyzed using ammonium hydroxide and 3% nickel(II) chloride solution, purified using dispersive solid phase extraction (d-SPE) kit, and then analyzed by LC-MS/MS. To prevent the matrix effect, all analytes were quantified using matrix-matched calibrations, as assessed by the determination coefficient (R^2) of the range from 0.9951-1.000. The LOD and LOQ were satisfactory for determining low residual levels of the pesticides in agricultural products. The accuracy and precision of the method were evaluated based on recoveries with five replicates at three fortification levels (LOQ, 10×LOQ and 50×LOQ). The mean recoveries of nereistoxin and nereistoxin-related pesticides in agricultural products were 75.3-108.0% with a coefficient of variation of 0.8-7.0%. All the optimized results were excellent, as assessed by the Ministry of Food and Drug Safety guidelines and the Codex Alimentarius Commission guidelines for pesticide residue analysis. This study provides fundamental data for setting the residue definition and maximum residue limits for nereistoxin and nereistoxin-related pesticides in agricultural products.

Keywords: Agricultural products, Analytical method, LC-MS/MS, Nereistoxin, Nereistoxin-related pesticides

<https://doi.org/10.5338/KJEA.2024.43.20>

Korean J. Environ. Agric. 2024, 43, 211-222

Received: October 7, 2024

Revised: October 12, 2024

Accepted: October 25, 2024

Published: November 11, 2024

Online ISSN: 2233-4173

Print ISSN: 1225-3537



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Introduction

Modern agriculture requires stringent quality control measures to ensure food safety. A synthetic pesticide is a chemical that works as a killer or a repellent to reduce, destroy, and kill pests such as insects, fungi, weeds, and other undesired organisms, that affect human health, agricultural crops, and the environment. The use of pesticides in agriculture helps increase the crop yield to meet the demands of the growing human population. The World Health Organization (WHO) reported that the consumption of the pesticides is increasing worldwide, especially in developed countries, where the usage of pesticides began in 1940. However, continuous and indiscriminate application of pesticides can lead to chronic health

problems in humans and destroy the environment and biodiversity. To avoid these dire consequences, synthetic pesticides should be replaced with natural organic pesticides [1,2].

Nereistoxin is a natural toxin isolated from the marine annelid worm, *Lumbriconereis heteropoda*. Nereistoxin exhibits a strong insecticidal activity by blocking the nicotinic acetylcholine receptor and inhibiting the conduction of Na^+ and K^+ through the endplate membrane, causing central nervous system disorders [3,4]. Cartap, bensultap, thiosultap and thiocyclam belong to the same group of pesticides that are metabolized to nereistoxin [3,5,6]. Nereistoxin-related pesticides, including cartap, bensultap, thiosultap and thiocyclam are widely used in agriculture because of their low mammalian toxicity and high effectiveness [4]. However, several studies have indicated that nereistoxin and nereistoxin-related pesticides pose potential toxicological risks to humans and animals, not only because of their acute toxicity, but also because of their potential ontogenetic developmental toxicity [7-10].

For the risk assessment and monitoring of pesticides, several foreign regulatory authorities, including the Codex Alimentarius Commission, US Environmental Protection Agency, and European Commission, have established maximum residue limits (MRL) and residue definitions for pesticides found in or on food products of plant and animal origin. For example, the European Union (European Commission, EC) stipulated the MRLs of cartap at 0.1 mg/kg in tea. Japan (Japan Food Chemical Research Foundation, JFCRF) mandated the MRLs of cartap, bensultap, and thiocyclam at 0.03-30 mg/kg in 36 crops, including rice, potato, and tea. According to the positive list system (PLS), when the MRL of pesticide have not established or when the pesticide is detected in domestic and imported foods other than agricultural products that are prescribed residual amount, the MRL should be less than 0.01 mg/kg. Currently, the MRL of cartap, bensultap, and thiocyclam of agricultural products in Korea is stipulated as 0.05-10 mg/kg, but the MRL of thiosultap has not been established. In addition, it is necessary to define the LOQ of nereistoxin and nereistoxin-related pesticides as 0.01 mg/kg for management of agricultural products that have not established MRL. Therefore, it is important to develop a simple and sensitive method for determining nereistoxin and nereistoxin-related pesticides to satisfy the PLS system.

Many analytical methods have been developed to determine the levels of nereistoxin and nereistoxin-related pesticides in biological fluids, soils, and foods of plant origin. Colorimetric assays based on gold nanoparticles [11,12] and instrumental methods such as GC-ECD [3], GC-MS [4], HPLC [13], and LC-HRMS [6], have been utilized for the identification and detection of nereistoxin and nereistoxin-related pesticides. Yang et al. [5] developed an analytical method for detecting nereistoxin and nereistoxin-related pesticides in foods of animal origin using hydrophilic interaction chromatography-mass spectrometry. However, an analytical method with satisfactory results for five agricultural products (hulled rice, potato, soybean, mandarin, and green pepper) is required as an official regulatory method in Korea. Accordingly, in this study, an analytical method that can determine nereistoxin and nereistoxin-related pesticides in five crops was developed using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Standard, reagents and samples

The pesticide standard of nereistoxin oxalate (98.0%) was purchased from Wako (Tokyo, Japan). Further, four nereistoxin-related pesticides, including cartap hydrochloride (97.5%), bensultap (98.1%), thiosultap-sodium (99.7%), and thiocyclam hydroxalate (95.0%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). HPLC-grade acetonitrile and methanol were procured from Merck (Darmstadt, Germany), and hydrochloric acid was obtained from FLUKA (Charlotte, US). Formic acid, ammonium hydroxide (NH_3 content: 28-30%), L-cysteine, and nickel(II) chloride were purchased from Sigma Aldrich (Buchs, Switzerland). A d-SPE kit (Part No. PM5EN) was purchased from CHROMAtific (Cacilienweg, Germany) and a PTFE syringe filter (0.2 $\mu\text{m} \times 13 \text{ mm}$) was obtained from Teknokroma (Barcelona, Spain). All the agricultural samples used to develop

the method were purchased and were not treated with pesticides. Five food samples, viz. mandarin, potatoes, soybeans, green peppers, and hulled rice were purchased from a local supermarket (E-mart, homeplus) in the city of Cheongju. The dry hulled rice and soybean samples were crushed with a blender and then filtered through a standard sieve of 420 μm , whereas the potato, mandarin, and green pepper samples were chopped and homogenized. The processed samples were placed in a plastic container and stored at -50°C in a freezer until use.

Preparation of stock solutions and standard solutions

Stock solutions of cartap, bensultap, thiosultap, and thiocyclam were prepared in methanol at concentration of 1,000 mg/L, respectively. On the other hand, a stock solution of nereistoxin was prepared in methanol at concentration of 100 mg/L. Further, calibration solutions were prepared by serially diluting the 2.5 mg/L stock solution of nereistoxin with acetonitrile to obtain solutions with concentrations of 0.01, 0.025, 0.05, 0.1, 0.25, and 0.5 mg/L. The matrix-matched solutions were prepared by diluting 100 μL aliquots of the calibration solutions with 900 μL of the blank samples to include more than 90% of the matrix. Standard solutions of nereistoxin and nereistoxin-related pesticides for the recovery test were prepared by diluting the corresponding stock solution with acetonitrile to concentrations of 12.5, 2.5, and 0.25 mg/L. Matrix-matched solutions of nereistoxin-related pesticides were prepared as described for the parent compound (i.e., nereistoxin). The stock solutions were stored at -20°C in amber glass vials in refrigerator, whereas the standard solutions were freshly prepared before each analysis.

Instrument conditions

Nereistoxin and nereistoxin-related pesticides were analyzed using a liquid chromatograph (Acquity UPLC, Milford, MA, USA) equipped with a tandem mass spectrometer (Xevo TQ-S, Milford, MA, USA) and were separated on a XBridge[®] Amide 3.5 μm column (2.1 mm i.d. $\times$ 100 mm L., 3.5 μm). The column temperature was maintained at 40°C . The mobile phase was a gradient system that started at 90% of acetonitrile (A) mixed with 10% of water with 5 mM ammonium formate, 0.1 formic acid (B). The linear mobile phase was maintained at 10% of B (0-0.5 min), increased to 30% of B (0.5-4 min), and then to 40% of B (4-5 min), held at 40% of B (5-7 min), and finally decreased to 10% of B (7.0-7.1 min), and held there (7.1-10.0 min). The flow rate and injection volume were 0.2 mL/min and 2 μL , respectively. An electrospray ionization (ESI) source was operated in the positive-ion mode for nereistoxin. The capillary voltage was 3 kV, and the source and desolvation temperatures were controlled at 150 and 500°C , respectively. The cone and desolvation gas flows were 150 and 1,000 L/hr, respectively. The multiple reaction monitoring (MRM) mode was selected for simultaneous analysis. Analytical condition and selected-ion for detection of these chemical were described more detail in Table 1.

Table 1. MRM (multiple reaction monitoring) conditions of nereistoxin for analysis in ESI positive mode

Compound	Molecular weight (g/mol)	Exact mass (g/mol)	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	Collision Energy (eV)	Cone Voltage (V)
Nereistoxin	149.3	149.03	3.1	150	105 ^a	16	22
					61	21	
					86	11	

^a Quantification ion.

Sample preparation

To analyze nereistoxin and nereistoxin-related pesticides in the five agricultural samples, each processed sample (5 g) was weighed into a 50 mL centrifuge tube. Dry samples (Hulled rice and soybeans) were wetted with 10 mL of water for 30 min. Next, 10 mL of a 2% L-cysteine solution in 0.05 N HCl was added to the tube, and then shaken for 10 min. After extraction,

0.5 mL of a 3% nickel(II) chloride (NiCl_2) solution and 1 mL of ammonium hydroxide were added to the sample. The tubes were capped loosely, and the samples were placed in a water bath at 70°C for 10 min. Then, they were maintained at room temperature to cool them. Thereafter, the samples were centrifuged at 4,000 rpm for 10 min at 4°C. Subsequently, 1 mL of the supernatant was added to a 2 mL tube containing 150 mg of anhydrous magnesium sulfate (MgSO_4) and 25 mg of a primary secondary amine (PSA). Then, the mixture was vortexed for 30 s and centrifuged for 10 min at 4,000 rpm and 4°C. Subsequently, the supernatant was collected and filtered through a syringe filter (PTFE, 0.2 μm $\times$ 13 mm).

Method validation

The method was validated by verifying the performance characteristics of the analytical method, such as the selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision. The validation results were assessed using the MFDS guidelines (Guidelines on standard procedures for preparing analysis method, 2016) on the standard procedures for developing an analysis method and the Codex Alimentarius Commission guidelines (CAC/GL 40-1993, 2010) for pesticide residue analysis.

Matrix effects (ME) are caused by the interaction of the target compound with the co-eluting components of the matrix. They can be assessed by calculating the average values of the peak area difference between the compound spiked into the blank sample and the compound dissolved in pure solvent and assessed in terms of ion suppression (loss in response) or ion enhancement (increase in response) [14,15]. ME(%) of nereistoxin were calculated as follows.

$$ME(\%) = \frac{(\text{Peak area of the standard a blank sample} - \text{Peak area of the standard in pure solvent})}{\text{Peak area of the standard in pure solvent}} \times 100$$

Results and Discussion

Optimization of LC-MS/MS conditions

The MS/MS parameters were optimized only for nereistoxin, because nereistoxin-related pesticides, including cartap, bensultap, thiosultap, and thiocyclam, are converted to nereistoxin via hydrolysis. The standard solutions were directly injected into the mass detector at a constant rate (10 $\mu\text{L}/\text{min}$) to optimize the precursor ion and product ions and thus obtain the optimal cone voltage and collision energy, which maximize the signal intensity. The mode multiple reaction monitoring (MRM) mode was used to quantify and identify the compounds. First, the intensity of an abundant protonated ion, $[\text{M}+\text{H}]^+$ (m/z 150) selected as the precursor ion was recorded for nereistoxin in the full-scan mode during the electrospray ionization process. Subsequently, the collision energy was gradually increased from 5 to 50 V to obtain fragment ions with higher intensities from the precursor ion. Three peaks with high m/z values of 105, 61, and 86 and abundances appeared in the fragment ion spectrum; therefore, these were utilized as the monitoring ions for quantification and identification.

Optimization of mobile phases

Different mobile phases were compared to achieve maximum sensitivity and resolution of nereistoxin. When 5 mM ammonium formate, 0.1% formic acid in methanol and 5 mM ammonium formate, 0.1% formic acid in water were used, the shape of the peak was even, but the base line was raised. When methanol was changed to acetonitrile under the same conditions, the base line was flat, but the shape of the peak was uneven. To improve the peak shape, 0.1% formic acid in acetonitrile and acetonitrile were analyzed with 5 mM ammonium formate, 0.1% formic acid in water. As a result, the shape of the peak was sharper and more sensitive when acetonitrile was used than when acetonitrile containing formic acid was used. Finally, the mobile phase decided to use acetonitrile and 5 mM ammonium formate, 0.1% formic acid in water (Fig. 1).

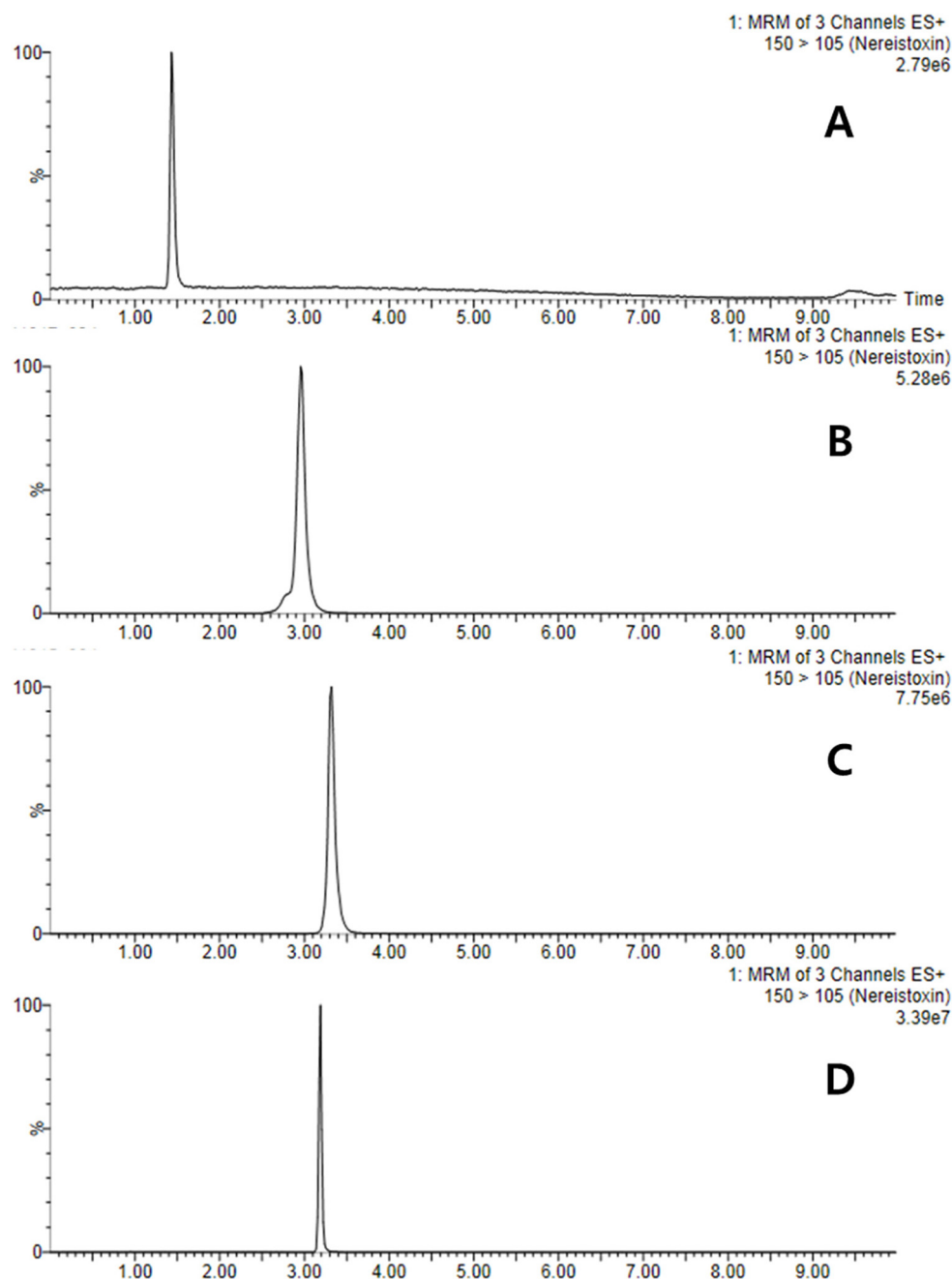


Fig. 1. LC-MS/MS chromatogram of standard solution of nereistoxin according to the different organic solvent with 5 mM ammonium formate and 0.1% formic acid in water (A) 5 mM ammonium formate and 0.1% formic acid in methanol, (B) 5 mM ammonium formate and 0.1% formic acid in acetonitrile, (C) 0.1% formic acid in acetonitrile (D) acetonitrile.

Optimization of sample extraction

Among the nereistoxin-related pesticides, cartap, thiosultap, and thiocyclam are polar and remain stable in acidic solutions. However, they are easily hydrolyzed to nereistoxin in insects or in alkaline solutions [3]. On the other hand, bensultap is moderately polar and remains stable in acidic media, and it is poorly extracted into acetonitrile and is easily hydrolyzed to nereistoxin monoxide under aqueous conditions [5]. Inoue and Yamamoto suggested the conversion of bensultap into nereistoxin disulfide in an acidic L-cysteine solution for achieving a higher extraction efficiency because of its incomplete hydrolysis to nereistoxin monoxide and partial absorption by the sample [13]. The metabolic pathways of nereistoxin and nereistoxin-related pesticides (cartap, bensultap, thiosultap, and thiocyclam) are illustrated in Fig. 2. As shown, nereistoxin disulfide can be converted to ner-

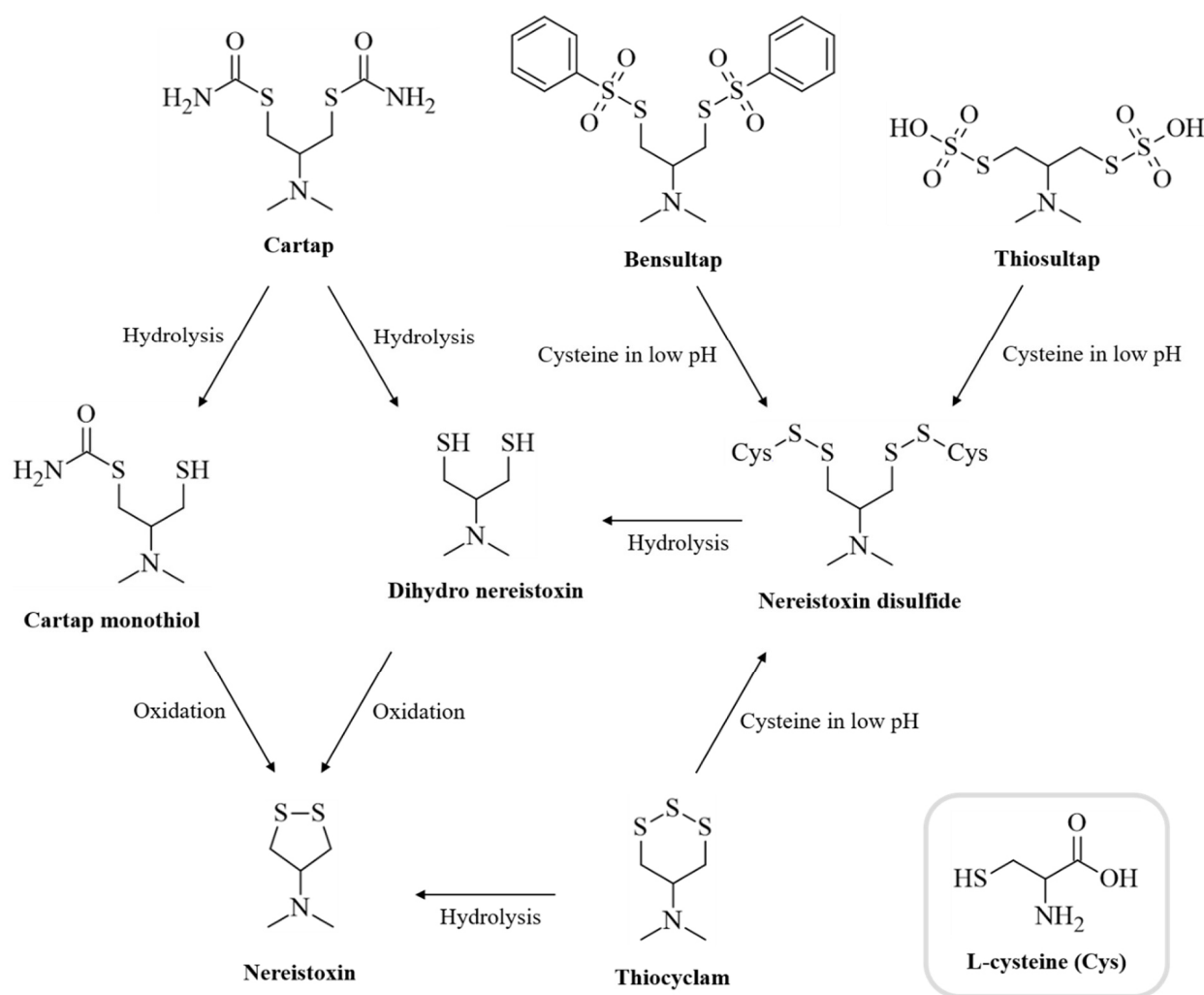


Fig. 2. Metabolism of nereistoxin and above nereistoxin-related pesticides.

eistoxin by the addition of ammonium hydroxide after (method A) or before (method B) the separation of the extract from the residue. L-cysteine is comparatively stable in acidic aqueous solutions but easily oxidizes to cystine in a basic aqueous solution. Because cystine is poorly soluble in water compared to L-cysteine, the basification of the extract by the addition of ammonium hydroxide causes the conversion of L-cysteine to cystine, which precipitates out. Therefore, in method A, a second filtration step prior to the clean-up by liquid-liquid partitioning becomes necessary. In method B, the precipitated cystine can be removed from the residue by centrifugation during the extraction step. The recovery of bensultap is generally better with method B than with method A [13].

To optimize the extraction conditions, the extraction efficiency was evaluated according to the concentrations of L-cysteine (1, 2, and 3%) and HCl (0.01, 0.02, and 0.05 N) in the extraction solution. Nereistoxin and nereistoxin-related pesticides were added to soybean samples to obtain a spike concentration of 0.01 mg/kg. Then, the samples were extracted with 10 mL of an L-cysteine solution (1, 2, or 3%) in 0.02 N HCl. Thereafter, the resulting mixture was shaken for 10 min and 0.5 mL of 3% nickel(II) chloride solution and 1 mL of ammonium hydroxide were added. Subsequently, the tubes were capped loosely and placed in a water bath at 70°C for 10 min. Then, the samples were centrifuged for 10 min at 4°C and 4,000 rpm. The mean recoveries at different L-cysteine concentrations in the extraction solutions are shown in Table 2. An L-cysteine concentration of 2% in the extraction solution (0.02 N HCl) was sufficient to obtain a high recovery and low relative standard deviations of nereistoxin from soybeans.

Then, the extraction efficiency was evaluated by varying the HCl concentration (0.01, 0.02, and 0.05 N) in the extraction solution. Nereistoxin and nereistoxin-related pesticides were added to the soybean sample to obtain a spike concentration of 0.01 mg/kg. The samples were then extracted with 10 mL of a HCl solution (0.01, 0.02, or 0.05 N) with 2% L-cysteine. The following process was the same as that described above. The mean recoveries at different HCl concentrations in the extraction solution are shown in Table 3. The results indicate that 2% cysteine in 0.05 N aqueous HCl (91.7-96.5%) is sufficient for obtaining a higher recovery than other two solutions in soybean samples.

Table 2. Extraction efficiency of nereistoxin and nereistoxin-related pesticide in soybeans according to L-cysteine concentration in the extraction solution (0.02 N HCl)

Compound	Recovery±RSD ^a (%)		
	1% L-cysteine	2% L-cysteine	3% L-cysteine
Cartap	92.9±3.5	92.0±4.2	85.2±1.3
Bensultap	85.8±0.1	86.2±1.8	76.8±3.4
Thiosultap	87.5±4.8	85.0±0.8	80.9±0.2
Thiocyclam	88.6±2.2	88.2±2.2	84.7±1.4
Nereistoxin	85.8±3.1	88.0±0.7	93.5±11.9

^a Mean values of 3 times repetitions with relative standard deviation.

Table 3. Extraction efficiency of nereistoxin and nereistoxin-related pesticide in soybeans according to HCl concentration in the extraction solution ([L-cysteine]=2%)

Compound	Recovery±RSD ^a (%)		
	0.01 N HCl	0.02 N HCl	0.05 N HCl
Cartap	92.3±2.4	91.8±0.3	92.7±0.1
Bensultap	91.4±1.1	90.9±0.3	96.5±1.1
Thiosultap	90.8±3.1	87.3±1.3	93.2±4.3
Thiocyclam	91.4±0.6	91.6±2.1	93.6±1.5
Nereistoxin	88.8±2.8	90.8±0.6	91.7±1.7

^a Mean values of 3 times repetitions with relative standard deviation.

Optimization of the conversion conditions

Nereistoxin-related pesticides, including cartap, bensultap, thiosultap, and thiocyclam, degrade to nereistoxin when hydrolyzed. Therefore, the recovery of bensultap was evaluated by fortifying it in different samples. Cartap, bensultap, thiosultap, and thiocyclam were catalytically converted to nereistoxin under alkaline conditions. To vary the alkalinity of the solution, 0.5, or 1.0 mL of NH₄OH was added. In addition, 0.5 or 1.0 mL of a NiCl₂ solution (3%) was added to accelerate the reaction. The recoveries were significantly higher when 0.5 mL of NH₄OH and 1.0 mL of the NiCl₂ solution were added (99.1-105.1%)(Table 4).

The time and temperature of the conversion process also significantly affect the conversion efficiency. The recoveries of nereistoxin and nereistoxin-related pesticides in soybeans were compared after conversion for 10, 30, and 60 min at room temperature (25°C) and 70°C. Compared to each conversion temperature, the recoveries were higher when left for 30 min at room temperature and 10 min at 70°C. Finally, the time and temperature of conversion process were selected 10 min and 70°C (94.1-107.1%), respectively, because the recoveries were significantly higher than that at room temperature for 30 min (85.8-94.6%) (Table 5).

Table 4. Extraction efficiency of nereistoxin and nereistoxin-related pesticide in soybeans according to the ratio of NH_4OH and NiCl_2

Compound	Catalyst	Recovery $\pm$ RSD ^a (%)	
		NH_4OH 0.5 mL	NH_4OH 1.0 mL
Cartap	NiCl_2 0.5 mL	89.4 $\pm$ 2.6	101.7 $\pm$ 6.6
Bensultap		75.0 $\pm$ 0.3	101.0 $\pm$ 0.1
Thiosultap		82.6 $\pm$ 2.1	105.1 $\pm$ 0.1
Thiocyclam		86.3 $\pm$ 0.7	102.4 $\pm$ 1.7
Nereistoxin		80.9 $\pm$ 1.1	99.1 $\pm$ 1.9
Cartap	NiCl_2 1.0 mL	95.2 $\pm$ 1.0	97.6 $\pm$ 1.4
Bensultap		90.7 $\pm$ 0.7	92.5 $\pm$ 0.3
Thiosultap		97.6 $\pm$ 3.0	99.1 $\pm$ 0.1
Thiocyclam		104.7 $\pm$ 0.1	95.4 $\pm$ 2.2
Nereistoxin		100.4 $\pm$ 9.3	99.6 $\pm$ 1.2

^a Mean values of 3 times repetitions with relative standard deviation.

Table 5. Extraction efficiency of nereistoxin and nereistoxin-related pesticide in soybeans according to the conversion temperature and time

Compound	Temperature	Recovery $\pm$ RSD ^a (%)		
		10 min	30 min	60 min
Cartap	25°C	82.3 $\pm$ 3.2	94.6 $\pm$ 0.8	88.8 $\pm$ 1.3
Bensultap		79.2 $\pm$ 0.3	85.8 $\pm$ 0.8	83.8 $\pm$ 4.9
Thiosultap		87.2 $\pm$ 7.8	92.1 $\pm$ 2.1	88.0 $\pm$ 2.3
Thiocyclam		81.8 $\pm$ 0.3	94.0 $\pm$ 0.5	86.0 $\pm$ 2.4
Nereistoxin		79.6 $\pm$ 2.8	92.2 $\pm$ 0.3	88.7 $\pm$ 0.4
Cartap	70°C	98.2 $\pm$ 1.9	90.7 $\pm$ 1.5	88.8 $\pm$ 0.9
Bensultap		96.3 $\pm$ 0.8	86.8 $\pm$ 1.2	83.3 $\pm$ 0.8
Thiosultap		107.1 $\pm$ 2.0	89.2 $\pm$ 0.1	89.2 $\pm$ 1.6
Thiocyclam		98.9 $\pm$ 1.5	88.4 $\pm$ 1.0	93.3 $\pm$ 1.3
Nereistoxin		94.1 $\pm$ 1.1	87.2 $\pm$ 1.5	90.6 $\pm$ 0.7

^a Mean values of 3 times repetitions with relative standard deviation.

Optimization of the d-SPE (dispersive-Solid Phase Extraction) procedure

The purification efficiency was examined using standard solutions and the d-SPE kits mixed with anhydrous MgSO_4 (magnesium sulfate anhydrous), PSA (primary secondary amine), C_{18} (octadecyl bonded silica), and GCB (graphitized carbon black). Typically, PSA, C_{18} , GCB and anhydrous MgSO_4 are used as sorbents in QuEChERS. PSA is a weak anion exchanger that can remove fatty acids, sugars, and other components that can form hydrogen bonds, whereas C_{18} can be used to remove of lipids and nonpolar interfering substances. GCB can efficiently remove pigments, particularly chlorophyll. Anhydrous MgSO_4 was used to absorb the water residue in the solvent to render the final acetonitrile extracts less polar and cause the precipitation of certain polar matrix co-extracts [20]. The supernatant (1 mL) was taken in a 2 mL tube containing the d-SPE kits components mixed with MgSO_4 , PSA, C_{18} , and GCB. As shown in Table 6, the best recovery was 99.0% obtained using 150 mg of MgSO_4 and 25 mg of PSA, which was closest to 100%.

Table 6. Recovery results of nereistoxin using the d-SPE kit

Sorbent	Recovery $\pm$ RSD ^a (%)
MgSO ₄ 150 mg, PSA 25 mg	99.0 $\pm$ 1.8
MgSO ₄ 150 mg, C ₁₈ 25 mg	89.3 $\pm$ 1.8
MgSO ₄ 150 mg, GCB 2.5 mg	96.0 $\pm$ 2.6
MgSO ₄ 150 mg, PSA 25 mg, C ₁₈ 25 mg	89.0 $\pm$ 1.2
MgSO ₄ 150 mg, PSA 50 mg, C ₁₈ 50 mg	62.7 $\pm$ 1.0
MgSO ₄ 150 mg, PSA 25 mg, GCB 2.5 mg	103.3 $\pm$ 1.4
MgSO ₄ 150 mg, PSA 25 mg, GCB 7.5 mg	98.7 $\pm$ 2.3
MgSO ₄ 150 mg, C ₁₈ 25 mg, GCB 2.5 mg	85.7 $\pm$ 1.1
MgSO ₄ 150 mg, PSA 50 mg, C ₁₈ 50 mg, GCB 7.5 mg	54.2 $\pm$ 1.7

^a Mean values of 3 times repetitions with relative standard deviation.

Method validation

To exam the analytical performance of developed method, such as the selectivity, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision was investigated.

Matrix effects values exceeding 20% indicate signal enhancement, whereas ME values less than -20% indicate signal suppression. ME values between -20% and 20% indicate negligible effect of the matrix [21]. Otherwise, the ME should be addressed in the calibration step, according to EU SANTE/12682/2019. Table 7 summarizes the ME and linearity of nereistoxin in the five agricultural samples. Ion suppression was observed in the analysis of nereistoxin in all crops, and the ME was below -80%. Therefore, matrix-matched calibrations were adopted for accurate quantification in this study.

Selectivity was assessed by comparing the chromatograms of the standard solutions, blank samples, and the samples spiked with the target compounds. The comparison of the chromatograms of the standard confirmed that highly selective analysis was realized without any interfering peaks at the retention times of the target analytes (Fig. 3). The linearity of the matrix-matched

Table 7. Matrix effects (ME), linearity, and recovery results for nereistoxin and four nereistoxin-related pesticides in five agricultural products

Sample	Fortification levels (mg/kg)	Recovery $\pm$ RSD ^a (%)					Linearity (R ²)	ME (%)
		Cartap	Bensultap	Thiocyclam	Thiosultap	Nereistoxin		
Hulled rice	0.01	88.5 $\pm$ 1.1	83.4 $\pm$ 4.0	75.3 $\pm$ 4.4	86.6 $\pm$ 2.6	93.3 $\pm$ 2.9	0.9993	-84.9
	0.1	100.9 $\pm$ 1.6	85.6 $\pm$ 1.9	106.6 $\pm$ 2.1	97.5 $\pm$ 0.8	103.6 $\pm$ 5.0		
	0.5	104.4 $\pm$ 2.4	93.9 $\pm$ 1.6	102.6 $\pm$ 2.1	107.3 $\pm$ 0.8	99.1 $\pm$ 2.3		
Potato	0.01	92.9 $\pm$ 7.6	89.1 $\pm$ 1.2	107.1 $\pm$ 3.5	90.9 $\pm$ 4.4	91.8 $\pm$ 3.5	0.9978	-84.8
	0.1	85.7 $\pm$ 2.1	91.5 $\pm$ 2.2	91.2 $\pm$ 1.7	92.4 $\pm$ 1.8	97.1 $\pm$ 2.0		
	0.5	79.1 $\pm$ 2.6	97.6 $\pm$ 0.9	89.3 $\pm$ 3.6	108.0 $\pm$ 1.6	89.6 $\pm$ 1.7		
Soybean	0.01	81.9 $\pm$ 1.4	86.7 $\pm$ 1.8	86.1 $\pm$ 5.2	81.3 $\pm$ 4.7	76.3 $\pm$ 5.1	0.9995	-81.3
	0.1	91.6 $\pm$ 6.6	85.8 $\pm$ 4.2	96.8 $\pm$ 7.0	94.3 $\pm$ 4.3	81.5 $\pm$ 5.2		
	0.5	84.9 $\pm$ 3.2	83.7 $\pm$ 2.9	94.8 $\pm$ 1.5	93.8 $\pm$ 2.2	85.4 $\pm$ 1.5		
Mandarin	0.01	103.2 $\pm$ 2.5	94.2 $\pm$ 2.1	107.2 $\pm$ 1.3	102.1 $\pm$ 1.2	98.9 $\pm$ 1.7	0.9997	-83.7
	0.1	99.1 $\pm$ 1.8	75.3 $\pm$ 1.2	102.8 $\pm$ 3.0	99.4 $\pm$ 1.8	97.3 $\pm$ 4.4		
	0.5	93.9 $\pm$ 1.7	83.7 $\pm$ 3.1	95.0 $\pm$ 3.7	95.9 $\pm$ 2.6	91.0 $\pm$ 1.0		
Green pepper	0.01	104.6 $\pm$ 3.8	88.8 $\pm$ 4.7	103.1 $\pm$ 2.3	104.5 $\pm$ 1.1	97.8 $\pm$ 3.1	1.000	-82.1
	0.1	102.7 $\pm$ 2.6	80.2 $\pm$ 1.8	103.5 $\pm$ 1.9	103.5 $\pm$ 2.4	99.1 $\pm$ 2.6		
	0.5	103.1 $\pm$ 1.9	94.4 $\pm$ 2.7	100.4 $\pm$ 0.8	96.4 $\pm$ 3.3	92.7 $\pm$ 1.6		

^a Mean values of 5 times repetitions with relative standard deviation.

calibrations at 0.001-0.05 mg/L of nereistoxin was excellent, as assessed by the determination coefficient (R^2), which ranged from 0.9978 to 1.0000 for all agricultural samples. The LOD is defined as the smallest concentration of the analyte that can be clearly distinguished from zero, and LOQ is the lowest concentration of the analyte that can be quantitatively detected. The LOD and LOQ were calculated as three and ten times the signal-to-noise ratios (S/N), respectively. The LOD and LOQ of nereistoxin and nereistoxin-related pesticides were estimated to be 0.00075 and 0.0025 mg/kg, respectively. The accuracy and precision of the developed method were evaluated by fortifying the blank samples at three fortification levels (LOQ, 10×LOQ, and 50×LOQ) and dividing them into five groups, because nereistoxin-related pesticides degrades into nereistoxin upon hydrolysis. The accuracy of the method was assessed in terms of recovery by calculating the average of five replicates, and precision was estimated from the relative standard deviation (RSD) of the within-laboratory recovery analyses. The mean recoveries of nereistoxin and four nereistoxin-related pesticides in agricultural products are 75.3-108.0% with the RSD of 0.8-7.0% (Table 7). All the validated results demonstrate that the method is reliable for the simultaneous analysis of nereistoxin and nereistoxin-related pesticide residues in agricultural samples.

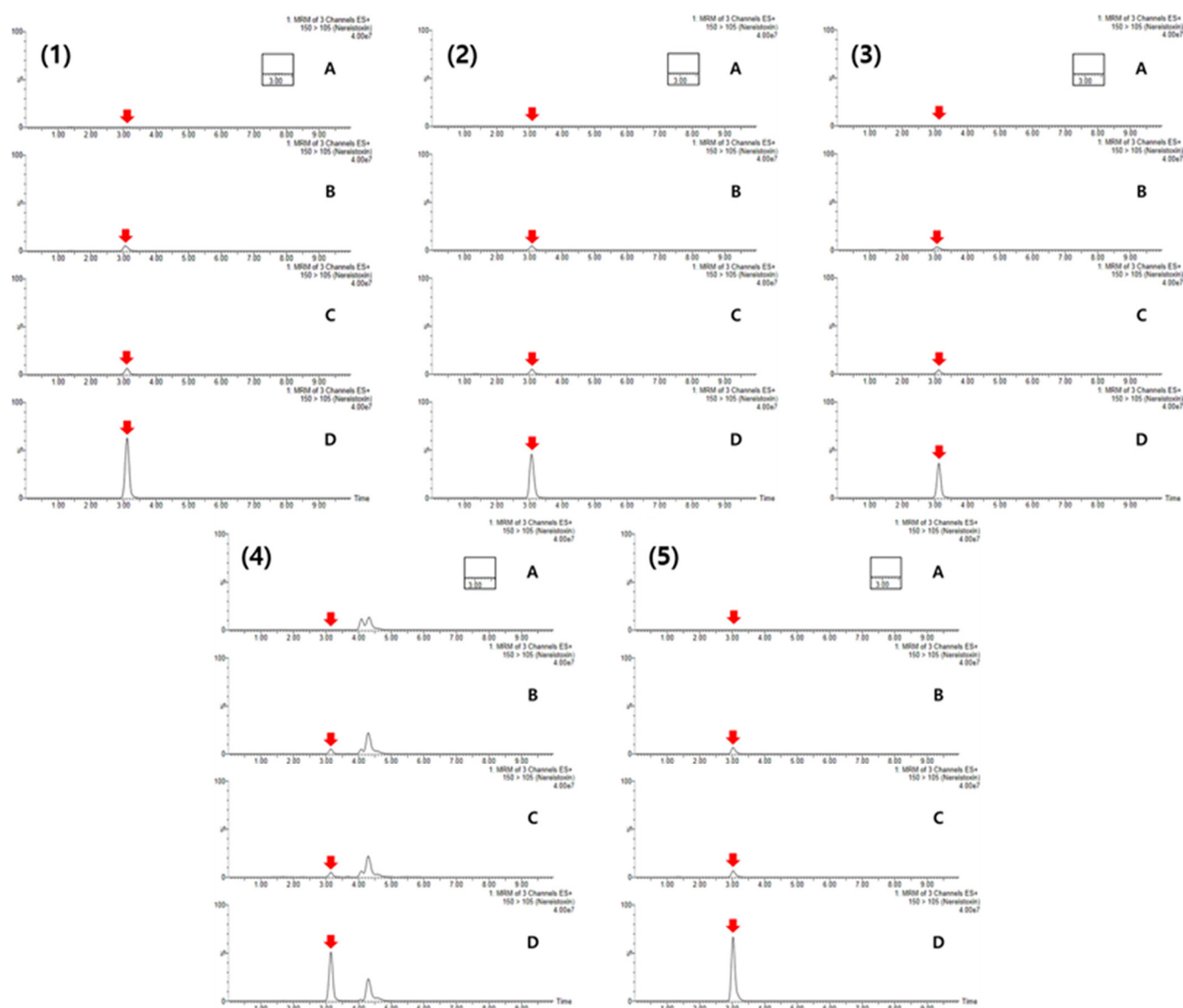


Fig. 3. Representative MRM chromatograms of nereistoxin corresponding to: (1) Hulled rice, (2) Potato, (3) Soybean, (4) Mandarin, (5) Green pepper, (A) blank sample, (B) matrix-matched standard at 0.0025 mg/L, (C) matrix-matched standard at 0.025 mg/L, and (D) recovery sample spiked at 0.01 mg/kg.

Conclusions

An effective and accurate analytical method for nereistoxin and nereistoxin-related pesticides in agricultural products was established and validated. Four nereistoxin-related pesticides, including cartap, bensultap, thiosultap, and thiocyclam were converted to nereistoxin using a conventional process. The d-SPE procedure combined with the LC-MS/MS ESI mode is convenient and simple for the analysis of cartap, bensultap, thiocyclam, thiosultap, and nereistoxin. The validation results satisfied the guidelines formally provided by the pesticide residue regulatory authorities. This method could be applied to monitor nereistoxin and nereistoxin-related pesticide residues in domestic and imported agricultural samples and can be utilized as a scientific reference for the establishment of residue definition and MRLs of cartap, bensultap, thiocyclam, thiosultap, and nereistoxin.

Data Availability: All data are available in the main text or in the Supplementary Information.

Author Contributions: Conceived and designed the research, C.Y.P. and J.M.L.; Collected the data, C.Y.P.; Performed the analysis, C.Y.P., S.J.L., and S.Y.G.; Wrote the first manuscript, C.Y.P.; Revised the manuscript, C.Y.P., S.J.L., S.Y.G., and J.M.L.; Supervision, M.O.E. and G.H.J.; All authors have read and agreed to the published version of the manuscript.

Notes: The authors declare no conflict of interest.

Acknowledgments: This study was supported by a grant (22191MFDS316) from the Ministry of Food and Drug Safety of Republic of Korea in 2023.

Additional Information:

Supplementary information The online version contains supplementary material available at <https://doi.org/10.5338/KJEA.2024.43.20>

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Peer review information Korean Journal of Environmental Agriculture thanks the anonymous reviewers for their contribution to the peer review of this work.

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