

Effective Extraction Method for Determination of Neonicotinoid Residues in Tea

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ABSTRACT: Sample preparation using an absorbent for removal of polyphenols and a solid-phase extraction (SPE) cartridge for cleanup followed by high-performance liquid chromatography (HPLC) has been investigated for the simultaneous determination of eight neonicotinoid insecticides (dinotefuran, nitenpyram, thiamethoxam, imidacloprid, clothianidin, imidaclothiz, acetamiprid, and thiacloprid). After tea samples were soaked with water and extracted with acetonitrile, sample extracts were treated with an appropriate amount of polyvinylpyrrolidone (PVPP) to effectively remove polyphenols. The treated extract was cleaned up with a Carb-PSA cartridge. Neonicotinoid insecticides were eluted with acetonitrile from the cartridge and dried. The extract was redissolved with methanol/water (1:9, v/v) and analyzed by conventional HPLC coupled with an ultraviolet detector. The recoveries of eight neonicotinoid insecticides in tea samples were 71.4–106.6% at 0.1–1.0 mg kg⁻¹ spiked levels. Relative standard deviations were <10% for all of the recovery tests. The established method was simple, effective, and accurate and could be used for monitoring neonicotinoid insecticides in tea.

KEYWORDS: tea, acetonitrile extraction, polyphenol sorbants, solid-phase extraction, neonicotinoid insecticides, HPLC

INTRODUCTION

Neonicotinoids, the newest major class of insecticides, represent the fastest growing class of insecticides since the launch of pyrethroids. Among them, imidacloprid is the most sold insecticide worldwide.¹ Neonicotinoid insecticides act as antagonists of the insect nicotinic acetylcholine receptor and are active against numerous sucking and biting insects, including aphids, whiteflies, beetles, and some lepidoptera species.^{1,2} There are several commercialized neonicotinoids: imidacloprid, acetamiprid, nitenpyram, thiacloprid, thiamethoxam, clothianidin, and dinotefuran.³ Imidaclothiz, a new neonicotinoid insecticide produced in China, is increasingly used in *Camellia sinensis* cultivation because of its excellent properties for control of a green leafhopper, *Empoasca flavescens* Fabricius.^{4,5} As polar compounds, neonicotinoids can be easily released from dry tea leaves into the drinkable tea infusions. This can be a significant source of human exposure to these residues.^{6–10} The increasing public concern in recent years about the health risks from pesticide residues in food emphasizes the importance of tea quality and safety. To ensure consumer health and safety, many countries and international organizations have defined maximum residue levels (MRLs) for several neonicotinoids in tea. The MRLs of neonicotinoids in tea set by the European Union (EU) range from 0.05 to 20 mg kg⁻¹,^{11–16} and those set by Japan range from 10 to 50 mg kg⁻¹ (Table 1).¹⁷ Single- and multi-residue analytical methods for the neonicotinoid pesticides in food have been reported using conventional HPLC and the more sensitive and accurate HPLC-MS because of their low volatility.^{18–30}

Tea represents a complex matrix containing not only caffeine (2–4%) but also a high amount of polyphenols (20–40%), including flavonols, flavones, flavanones, and phenolic acids,³¹ which can easily be coextracted with target pesticides and have high molar absorbances in the UV–vis region that could

Table 1. Current Maximum Residue Limits for Neonicotinoid Insecticides in Tea^{11–17}

insecticide (residue definition)	MRLs in tea (mg kg ⁻¹)		
	EU	Japan	China
dinotefuran	/	25	/
nitenpyram	/	10	/
thiamethoxam (sum of thiamethoxam + clothianidin)	20	15	10
imidacloprid	0.05	10	0.5
clothianidin	0.7	50	/
imidaclothiz	/	/	/
acetamiprid	0.1	30	/
thiacloprid	10	30	/

¹The MRL of the insecticide has not been set.

interfere with the determination of neonicotinoid insecticides. These interfering peaks and the complicated extraction methods for HPLC make the analysis of neonicotinoid residues difficult.^{6–10} HPLC-MS^{26–28} techniques for the determination of neonicotinoids in tea have been used, but they often suffer from analyte ionization suppression or enhancement by coeluting compounds in the electrospray ionization (ESI) source, known as the matrix effect.³² Although there are some approaches that can alleviate the matrix effect, such as matrix-matched standards, it is difficult to obtain a “blank” tea (pesticide-free blank of same cultivar and that has undergone the same processing) to be used as a matrix-matched standard. In addition, HPLC-MS techniques are costly and may not yet

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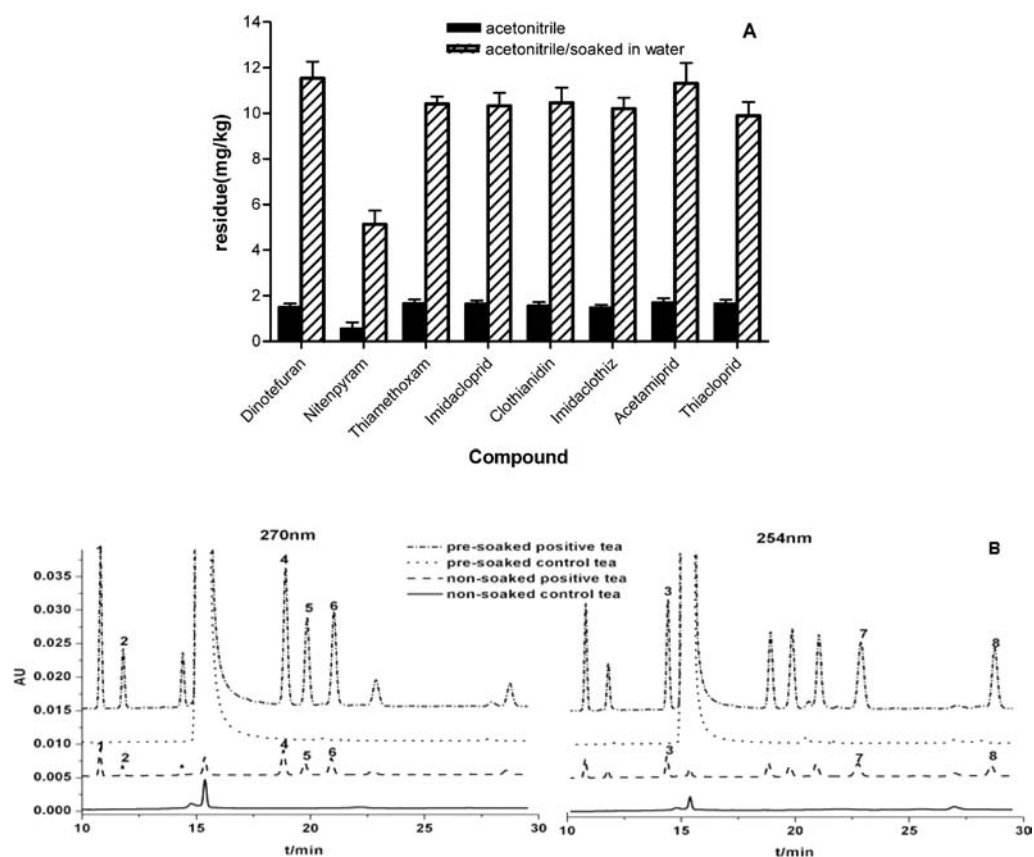


Figure 1. Eight neonicotinoid insecticides from treated tea samples that were extracted with ACN alone or after soaking in water: (A) residue levels (mg kg^{-1}) ($n = 3$); (B) chromatograms. Elution peaks for each neonicotinoid as numbered: 1, dinotefuran; 2, nitenpyram; 3, thiamethoxam; 4, imidacloprid; 5, clothianidin; 6, imidaclothiz; 7, acetamiprid; 8, thiacloprid.

be available as a routine method in a common analytical laboratory. A multi-residue analytical method for neonicotinoid insecticides has not been reported for tea samples with the HPLC method. The objective of this work was to develop an effective preparation method for the determination of eight neonicotinoid insecticides in tea samples.

MATERIALS AND METHODS

Apparatus. A Waters 1525 HPLC connected to a 2489 ultraviolet detector (Waters Corp., Milford, MA, USA) was used, equipped with a degasser and an autosampler.

Reagents. Certified neonicotinoid insecticide standards (dinotefuran, 98.6%; nitenpyram, 98.6%; thiamethoxam, 98.5%; clothianidin, 99%; imidacloprid, 98.0%; acetamiprid, 98.1%; thiacloprid, 98%) were obtained from Dr. Ehrenstorfer (Augsburg, Germany), and imidaclothiz solution ($100 \mu\text{g mL}^{-1}$ in acetonitrile, ACN) was purchased from Agro-environmental Protection Institute, Ministry of Agriculture (Tianjin, China). Stock standard solutions for seven insecticides (except imidaclothiz) were prepared in ACN at $500 \mu\text{g mL}^{-1}$. Working standard solutions were prepared by diluting the stock solution with methanol/water (1:9). Both solutions were stored at 4°C . Dual layer cartridges Envi-Carb II/PSA (500 mg of graphitized carbon black and 500 mg of primary–secondary amine; Supelco, Bellefonte, PA, USA) were used. A nitrogen evaporator (N-EVAP, Organomation, USA) and pulverizer (A11, IKA, Germany) were used. ACN and methanol were of HPLC grade and purchased from Tedia Co. (Fairfield, OH, USA). HPLC grade water was produced with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Polyvinylpyrrolidone (PVPP), AlCl_3 , PbAc_2 , sodium chloride, sodium hydroxide, and anhydrous sodium sulfate (dried at 550°C for 5 h and stored in desiccators) were of analytical grade.

Tea Samples. Positive tea samples (containing eight neonicotinoid insecticides) and blank tea samples (control) were made from field trials as follows: Field trials were carried out at the Tea Experimental Farm of Anhui Agricultural University at Hefei, Anhui province, China. Field trials consisted of a plot size area of $10 \times 1 \text{ m}^2$ for the control or separate treatment with neonicotinoid insecticides. The control plot was partitioned and isolated from the treated plot by leaving two untreated rows as guard rows. The treated trees were sprayed with 500 mL of aqueous solution containing eight neonicotinoid insecticide standard solutions ($10 \mu\text{g mL}^{-1}$ each) onto the fresh tea leaves. About 400 g of the fresh tea shoots (two leaves and a bud) were harvested on the second day after treatment from treated and control plots and brought to the Tea Experimental Factory, where they were processed in the laboratory's mini-manufacturing unit, using a conventional Chinese green tea manufacturing process.

Green tea samples purchased from supermarkets in Hefei, Anhui province, China, were used for blanks or spiked samples for recovery assays. Before the recovery assays, the samples were tested for the absence of neonicotinoid insecticides.

Tea samples (green and black tea) for application of the method were purchased from supermarkets in Hefei, Anhui province, China.

Sample Extraction. *PVPP Absorbent.* Dry tea samples were ground and sized by a 50 mesh sieve. A 1.0 g aliquot of sieved sample was weighed into a 150 mL conical flask with a stopper, soaked with 2 mL of water for 30 min, and then mixed with 20 mL of ACN. The mixture was homogenized for 1 min and then allowed to rest for 10 min. The extract was obtained by filtration (through Whatman no. 1 paper) into a 50 mL centrifuge tube. To this extract were added 2 g of anhydrous sodium sulfate and 1.5 g of PVPP. The sample was shaken by vortex for 2 min and then centrifuged at 6000 rpm for 5 min. A 5 mL aliquot of the supernatant, equivalent to 0.25 g of sample, was evaporated to 2 mL with a nitrogen stream at 40°C , ready for cleanup with SPE.

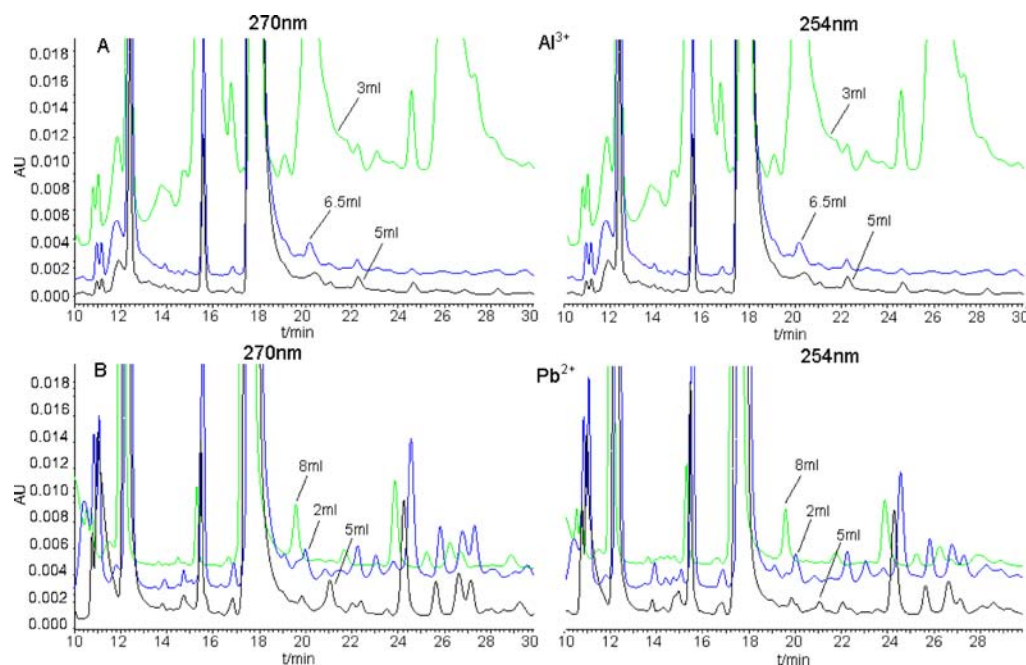


Figure 2. Chromatograms of the test solution pretreatment with (A) 1 mL of 20% AlCl_3 and different volumes of 1 mol^{-1} NaOH or (B) with different volumes of saturated PbAc_2 .

Metal Ion Precipitation. For Al^{3+} precipitation, tea sample was extracted with ACN and filtered as above and then mixed with 1 mL of 20% AlCl_3 , followed by 5 mL of 1 mol L^{-1} NaOH added to adjust the pH to 5.3 and then shaken together by vortex for 2 min. For Pb^{2+} precipitation, 6 mL of a saturated lead acetate (PbAc_2) solution was added and shaken; centrifugation was as for the method of Al^{3+} precipitation. A 5 mL aliquot of the extract, equivalent to 0.25 g of sample, was evaporated to 2 mL using a stream of nitrogen at 40°C , at which time it was ready for cleanup with SPE.

SPE Cleanup. Both types of extracts were subjected to solid-phase cleanup procedures. The concentrated extract in ACN (2 mL) was passed through an ENVI-Carb II/PSA SPE cartridge (preconditioned with 5 mL of ACN with 1 cm anhydrous sodium sulfate was added) with the aid of a vacuum or positive pressure and rinsed with an additional 6 mL of ACN. All combined eluants were evaporated to dryness in a water bath under a stream of N_2 . The residue was dissolved in 1 mL of methanol/water (10:90, v/v) and the extract, equivalent to 0.25 g of sample, was passed through a $0.45 \mu\text{m}$ pore size filter membrane, ready for injection into HPLC.

HPLC-UV Analysis. The HPLC analyses were performed using a Waters Alliance 2695 LC system, equipped with a degasser and an autosampler. Chromatographic separation was achieved on a Phenomenex Gemini C18 column ($250 \text{ mm} \times 4.6 \text{ mm}$, i.d., $5 \mu\text{m}$ particle size) at ambient temperature. The injection volume was $40 \mu\text{L}$, and the flow rate was 1.0 mL min^{-1} . The UV wavelength used in the experiment was either 270 nm for dinotefuran, nitenpyram, imidacloprid, clothianidin, and imidaclothiz or 254 nm for thiamethoxam, acetamiprid, and thiacloprid. The mobile phase was methanol (B) and water (A). The insecticides were separated with the following LC gradient program: 0–5 min, from 10 to 20% B; 5–10 min, from 20 to 30% B; 10–30 min, from 30 to 40% B; 30–31 min, return to 10% B; 31–40 min, equilibration of the LC system. Insecticides were identified according to the retention time, and quantification was based on peak area ratio of the target analyses divided by external standard. Spiked blank samples were used as standard to counteract the possible matrix effect, and the sample was spiked before extraction.

Method Performance. The sensitivity and precision of the method were evaluated by use of spiked tea samples. Recoveries and relative standard deviations (RSD) were determined for five replicates at three concentration levels (0.1, 0.5, and 1.0 mg kg^{-1}). The recovery was

quantified by addition of known levels of external standards to blank sieved sample. Spiked sample was allowed to stand for 0.5 h before extraction.

RESULTS AND DISCUSSION

Validation of Solvent Extraction Procedure. ACN is commonly reported for the extraction of residues of neonicotinoid insecticides^{6–10,26–30} in tea. The main difference between these reported extraction procedures using ACN is whether the tea was presoaked in water before extraction. Although the recoveries of eight neonicotinoid insecticides in spiked samples (extracted with ACN) were all excellent, the residues in the green tea samples made from the insecticide-treated tea plants varied greatly between presoaked and unsoaked methods. To compare the extraction efficiencies of these eight neonicotinoids from presoaked and unsoaked samples, the neonicotinoid-treated and control tea samples were extracted by ACN with three replicates each presoaked and unsoaked. Tea samples (1.0 g) were either soaked in water (2 mL) for 30 min and then extracted with ACN (20 mL) by homogenization for 1 min or directly extracted with the same volume of ACN by homogenization for 1 min without the water soaking step. These extracts were cleaned up with PVPP absorbent and SPE cartridges and then determined with HPLC. The result showed that the concentrations of the eight insecticides in the unsoaked tea samples were all lower than those of the soaked tea (Figure 1A). The chromatograms of positive and control samples are shown in Figure 1B. For eight neonicotinoid residues, the recoveries from tea sample without soaking were <80% of the amount recovered from the presoaked tea extracted with ACN. Longer homogenization time (3, 5 min) and greater volume of water (4, 6, 8, 10 mL) did not increase neonicotinoid residue extraction rates in tea samples. On the other hand, the content of nitenpyram decreased from 5.13 to 4.25 mg kg^{-1} when the soaking water volume increased from 2 to 10 mL. To sufficiently extract all eight residues from prepared tea, the following extraction

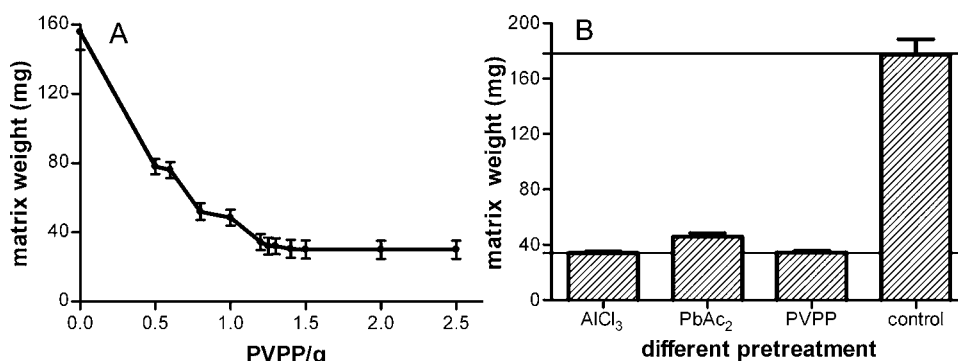


Figure 3. Comparison of matrix weight following pretreatment with (A) different amounts of PVPP or (B) PVPP (1.5 g), Al³⁺ (1 mL of 20% AlCl₃ and 5 mL of 1 mol L⁻¹ NaOH), Pb²⁺ (6 mL of a saturated PbAc₂ solution) or without pretreatment.

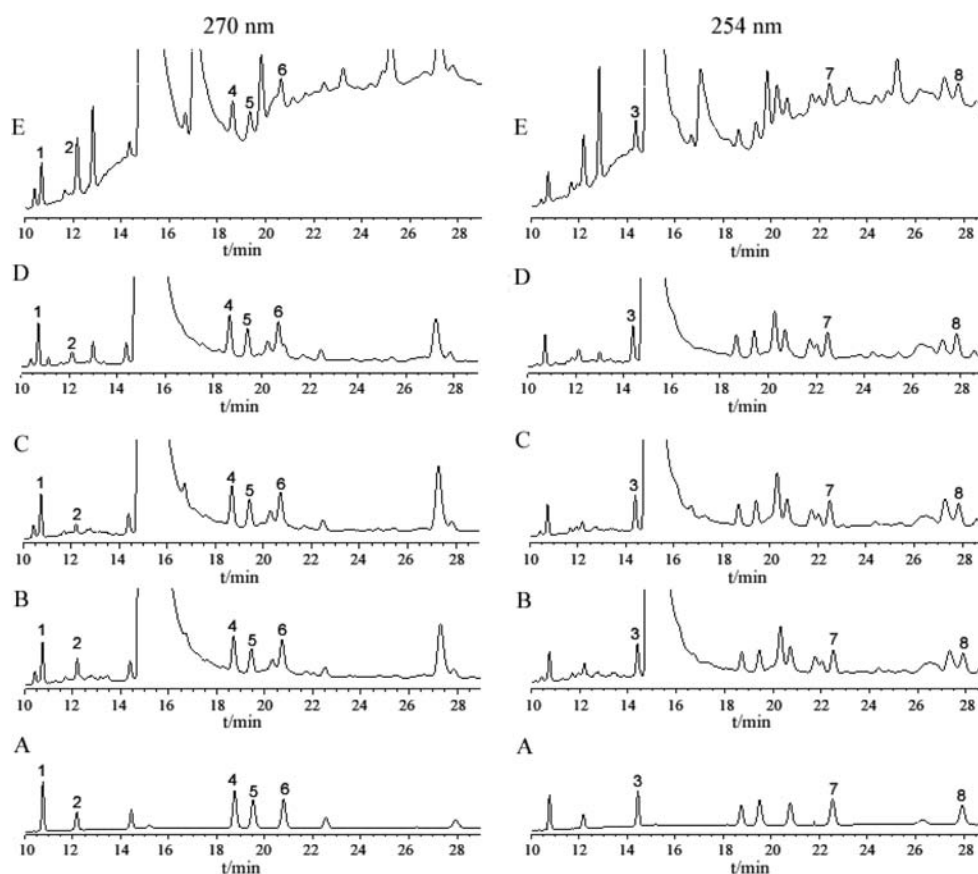


Figure 4. Elution chromatograms of eight neonicotinoid residues from tea extracts (spiked level of 0.5 mg kg⁻¹ for each residue) cleaned up by different precipitation/absorbance combined a same SPE pretreatment (B, PVPP; C, PbAc₂; D, AlCl₃) or without precipitation/absorbance pretreatment (E) and standard solutions (A) at 270 and 254 nm. Elution peaks for each neonicotinoid are numbered: 1, dinotefuran; 2, nitenpyram; 3, thiamethoxam; 4, imidacloprid; 5, clothianidin; 6, imidaclothiz; 7, acetamiprid; 8, thiacloprid.

procedure was proposed: tea sample (1.0 g) was soaked for 30 min with 2 mL of water before extraction with ACN.

Comparison of Different Precipitating Agents. In previous studies, metal ion compounds have been shown to precipitate polyphenols from aqueous tea extract, such as Al³⁺, Zn²⁺, Fe³⁺, Mg²⁺, Ba²⁺, Ca²⁺ and Pb²⁺.³⁴ Among these metal ion compounds, Al³⁺ and Pb²⁺ have excellent capacity for precipitation, although the precipitation capacity of Al³⁺ varies with the pH of tea extract. Other than metal ions, an inexpensive and excellent absorbent of polyphenols, PVPP, is often used to eliminate polyphenols from extract of plants, including tea.³⁵

To optimize the diminishment of polyphenol with Al³⁺ precipitation, filtered tea extract (20 mL, confirmed as blank for these insecticides, premixed with 1 mL of 20% AlCl₃) was mixed with different volumes (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 mL) of 1 mol L⁻¹ NaOH. A 5 mL aliquot of the extract (5 mL) was cleaned up with SPE cartridge and analyzed with HPLC. The effectiveness of the cleanup with different volumes of NaOH was evaluated through comparing matrix disturbance in the HPLC chromatograms of these samples. The addition of 5.0 mL of NaOH solution produced the minimum absorbance of disturbed peaks (Figure 2A), so 5.0 mL of 1 mol L⁻¹ NaOH was used for tests comparing Al³⁺ and Pb²⁺ precipitation.

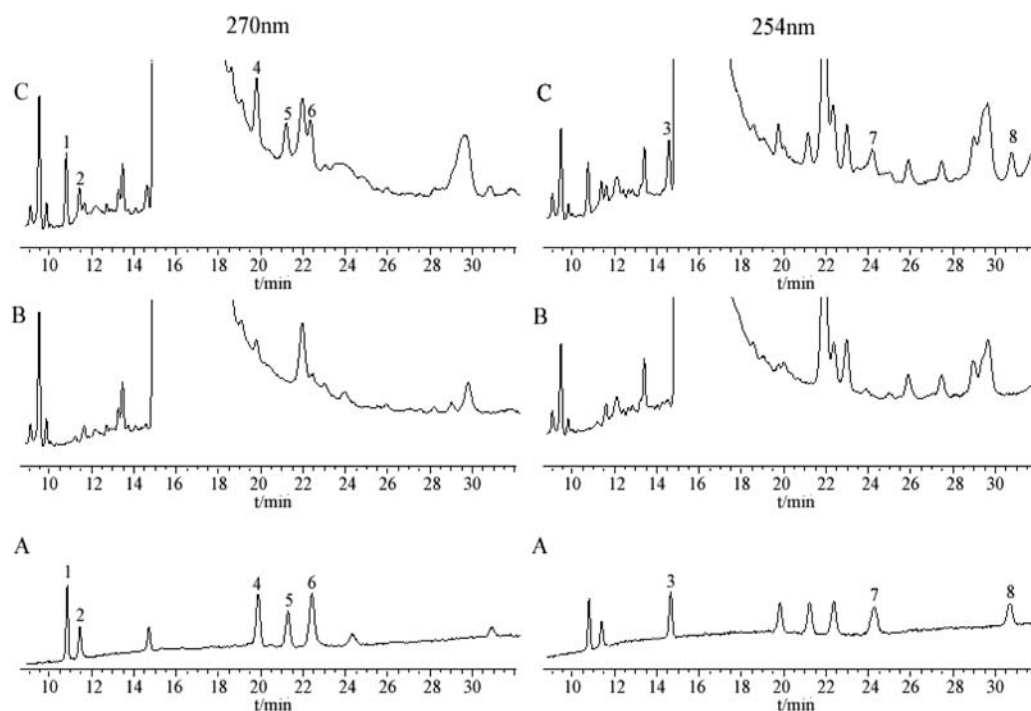


Figure 5. Chromatograms of standard (A), control matrix (B), and spiked tea samples (C). Each residue is at a spiked level of 0.1 mg kg^{-1} : 1, dinotefuran; 2, nitenpyram; 3, thiamethoxam; 4, imidacloprid; 5, clothianidin; 6, imidaclothiz; 7, acetamiprid; 8, thiacloprid.

To evaluate the capacity of Pb^{2+} to diminish polyphenol interference in tea extract, different volumes of the saturated PbAc_2 solution (2, 3, 4, 5, 6, 7, 8 mL) were added to each blank tea extract (following the above method of evaluation for AlCl_3). The cleanest baseline was detected when 5 mL of PbAc_2 solution was used (Figure 2B).

To investigate the absorbent capacity of PVPP, different amounts of PVPP (0–2.5 g) were added. Their capabilities to diminish the polyphenol disturbances were compared in tea matrix (Figure 3A). The dried matrixes were weighed after absorbance. The results showed that the average dried matrix weight (155.8 mg) was decreased when the amount of PVPP increased to 1.5 g (30.1 mg), but was not obviously changed as PVPP increased from 1.6 to 2.5 g. Therefore, 1.5 g of PVPP was used in our proposed method.

Using these above optimized methods, the capabilities of these three materials to diminish the polyphenol disturbances were compared in tea matrix. The dried matrixes were weighed after precipitation or absorbance ($n = 3$). The average matrix weight without polyphenol removal is nearly 5 times greater than those after a cleanup method (AlCl_3 , PbAc_2 , and PVPP, Figure 3B). The addition of a precipitating or absorbing agent greatly diminished a large quantity of compounds from the extract. Meanwhile, the cleanup capabilities of absorbing agents (PVPP) showed no obvious difference after the following SPE cleanup procedure (Figure 4).

Absorbance and SPE Cleanup. Well-established SPE procedures for insecticides have higher cleanup efficiencies and consume smaller amounts of organic solvents than conventional methods such as liquid–liquid partition or column chromatography. The method for imidacloprid residue detection in fruits, vegetables, and tea was established by our group using the ENVI-Carb II/PSA cartridge, which is compatible with different adsorbents (C18, NH_2 , Carb- NH_2).^{10,24,29,30} However, when using the ENVI-Carb II/PSA

SPE cartridge on tea samples following the conditions developed for optimum elution, the cleanup efficiency was low because coextractives were eluted with the target insecticides and resulted in high background and imprecise quantification, particularly for nitenpyram, imidaclothiz, and thiacloprid (Figure 4E). To determine multiple neonicotinoid residue levels in tea matrix, polyphenol absorption and removal were necessary. Pretreatment with the optimized AlCl_3 , PbAc_2 , or PVPP levels as determined above diminished the interference in the HPLC absorption chromatogram at both 270 and 254 nm (Figure 4B–D). The average recoveries for eight neonicotinoid insecticides from the tea samples at three spiked levels (0.1, 0.5, and 1.0 mg kg^{-1}) were $>70\%$. One limit is that the average recoveries of nitenpyram with precipitation agents (AlCl_3 and PbAc_2) were all $<50\%$, similar to those of Watanabe²⁰ and our previous work²⁴ for the determination of nine nicotinoid residues in vegetables and fruits. Through further investigation, it was determined that the lower recovery of nitenpyram is due to its lower partition coefficient in the organic (ACN) layer (data not shown) in the extraction procedure. Therefore, the absorbant agent PVPP (1.5 g) was used in our proposed method.

Although interfering compounds in tea extract were diminished in our proposed method, there remains an interfering peak in proximity to the peak of imidaclothiz at 270 nm (Figures 4 and 5, peak 6). The nature of this interfering compound, from the tea extract, will be investigated in our further research.

Linearity of Calibration Standards. For quantitative analysis, a calibration curve was obtained by analyzing insecticide standards at six different levels (0.02, 0.05, 0.1, 0.2, 0.5, and 1.0 mg L^{-1}). The UV detection was linear for the eight neonicotinoids from 0.02 to 1.0 mg L^{-1} with linear regression coefficients (R^2) >0.9990 .

Table 2. Recoveries and Relative Standard Deviations (RSDs) of Eight Neonicotinoid Insecticides in Spiked Tea Samples ($n = 5$)

insecticide	LOQ (mg kg ⁻¹)	spiked level 1 0.1 mg kg ⁻¹		spiked level 2 0.5 mg kg ⁻¹		spiked level 3 1.0 mg kg ⁻¹	
		mean ^a (%)	RSD (%)	mean ^a (%)	RSD (%)	mean ^a (%)	RSD (%)
dinotefuran	0.02	89.7	1.2	81.7	2.9	90.3	2.6
nitenpyram	0.1	71.4	7.8	83.4	6.7	78.5	8.0
thiamethoxam	0.05	78.9	2.6	102.2	2.1	101.5	2.6
imidacloprid	0.02	94.5	7.3	99.2	2.6	105.9	3.1
clothianidin	0.05	101.2	7.4	99.1	4.4	103.9	1.1
imidaclothiz	0.1	77.6	5.2	86.0	4.2	106.6	1.2
acetamiprid	0.05	83.7	1.4	99.0	1.6	95.1	0.8
thiacloprid	0.05	106.5	7.4	101.2	2.0	93.9	9.8

^aAverage of five replicates.

Precision and Accuracy. Intraday precision was examined by analysis of the same standard solutions at three different concentrations (high, medium, and low) on the same day;¹⁵ interday precision was determined by analysis of the same solutions three times over 7 days. The RSD values were 1.65 and 2.04%, respectively. Method accuracy and recovery were evaluated by addition of standard solutions in “blank” tea. Five portions of tea matrix were spiked with target compounds at three concentration levels: 0.1 mg kg⁻¹ (low, 1), 0.5 mg kg⁻¹ (intermediate, 2), and 1.0 mg kg⁻¹ (high, 3). The recoveries of eight neonicotinoid insecticides from tea sample ranged from 71.4 to 106.6%, with relative standard deviations (RSDs) of 1.1–9.8% (Figure 5; Table 2).

Detection and Quantification Limits. Limits of detection (LODs) were calculated from the peak intensity and blank levels in the recovery test. LODs were established by considering a value 3 times the background noise of the blank sample at the retention time of each pesticide. The limits of quantification (LOQs) were calculated as signal-to-noise ratio of 10 (S/N = 10). The LOQs obtained for each insecticide are shown in Table 2. For the neonicotinoids currently available to growers, imidacloprid and acetamiprid, the LOQs were 0.02 and 0.05 mg kg⁻¹, respectively, and are below the MRLs for tea set by both Japan and the European Union (EU).

Application of the Method to Commercial Samples. The developed method of sorbant pretreatment and SPE cleanup was applied to analyze the neonicotinoid insecticides in 53 tea samples (30 green tea samples and 23 black tea samples) collected from commercial teas prepared for export. Two samples positive for imidacloprid were detected, at 0.18 and 0.27 mg kg⁻¹. These measured concentrations were both below the MRLs for tea set by Japan (10–50 mg kg⁻¹) but above the EU MRL (0.05 mg kg⁻¹ for imidacloprid).

The reported method is effective, simple, and accurate. It is the first report that neonicotinoid extraction rates vary due to presoaking (vs nonsoaked tea) before ACN extraction using sample treated with insecticides in the field. It is also the first time that PVPP was investigated for use in diminishing the main interfering compounds (polyphenols) for pesticide residue determination in tea matrix. This treatment has proven useful for the simultaneous detection of eight neonicotinoid insecticides in tea samples. Furthermore, this pretreatment method could be used in the determination of multiple pesticide residues with LC-MS or LC-MS/MS, which also are disrupted by the matrix effect of tea samples.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation; PVPP, polyvinylpyrrolidone; PSA, primary–secondary amine

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