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Solid Phase Extraction and HPLC Analysis of Pymetrozine Residues in Green Tobacco Leaves

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A new method to determine the residues and persistence of pymetrozine in green tobacco leaves is developed. The pymetrozine residues levels were evaluated by extracting using acetonitrile/water, clean-up using two SPE cartridges and analyzing by HPLC. Average recoveries ranged from 97-99 %, with RSDs below 2.1 %. The limit of detection (LOD) was 0.005 μg mL^{-1}. In field trial, pymetrozine was treated with spraying and irrigation, respectively. Data indicated that higher residues of pymetrozine were found in 1-2 weeks after used the pesticide. After 4 weeks, the residues were lower than the guidance residue limit (GRLs). An analytical method for the determination of pymetrozine residues in green tobacco leaves has been developed. Pymetrozine poses low residues and transitory persistence in green tobacco leaves under the recommended dose.

Key Words: Pymetrozine, Tobacco, Solid-phase extraction, Residues, HPLC.

INTRODUCTION

Pymetrozine {1,2,4-triazin-3(2*H*)-one,4,5-dihydro-6-methyl-4-[(3-pyridylmethylene)amino]; IUPAC; Fig. 1}, a pyridine azomethine compound, is a novel insecticide with selective activity used for controlling homopteran insects such as tobacco whiteflies, aphids and planthoppers on a wide range of field, fruits and ornamental crops[1,2]. As a systemic chemical, pymetrozine is effective against xylem feeders and interrupts transmission of plant pathogens due to its mobility in the xylem tissue of plants[3]. In recent years, pymetrozine has been widely used in a variety of plants across the world. Especially, it is becoming available worldwide for the control of tobacco aphids and whiteflies in tobacco production. In general, it has a low acute toxicity to humans, birds, aquatic, organisms, mammals and bees[4,5]. However, the US environmental protection agency (EPA) has classified it as a "likely" human carcinogen because two types of cancerous tumors have been observed on the livers of two species of rat and mouse of both genders (liver benign

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hepatoma and carcinoma)[4]. Some countries, including the EU member states and Japan, have established MRLs for pymetrozine of 0.02-2 μg g^{-1} for vegetables and fruits[6,7]. The cooperation centre for scientific research relative to tobacco (CORESTA), an international cooperation organization, has developed a guidance residue level (GRLs) for pymetrozine of 1.00 μg g^{-1} in tobacco[8]. As such, determination of pymetrozine residues in tobacco leaves have aroused great concern in the tobacco industry.

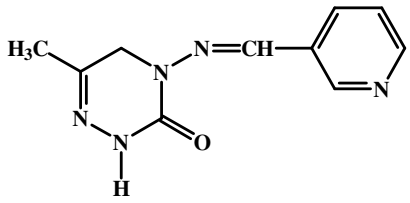

Fig. 1. Chemical structure of pymetrozine

A few methods for analyzing pymetrozine can be found in literature, such as enzyme immuno assay, liquid chromatography (LC) with ultraviolet (UV) or diode-array (DAD) detection, which were used[5,9] to analyzed samples of meat, milk, poultry, cured tobacco, *etc.*, but all these methods needed high cost in clean-up procedures and sometimes obtained unsatisfied results. Previous studies have investigated the pymetrozine residues in flue-cured tobacco leaves,[10] which clean up procedures was based on liquid-liquid extraction (LLE). The main disadvantages of LLE are that it is labour-intensive, time-consuming and require substantial quantities of high-purity organic solvents, which are toxic and somewhat costly[11,12]. Furthermore, the said analytical method is not suitable for the determination of pymetrozine in green tobacco leaves, due to it's containing more compounds such as alkaloid, pigments, lipid, proteins, pectin and so on. However, solid-phase extraction (SPE) is one of the simplest, most effective and versatile method for sample preparation, which is established in the analytical chemistry laboratory and has widely replaced classical LLE[13,14].

The aim of this work is to develop a sensitive and selective HPLC-UV method to determine low levels of pymetrozine residues in green tobacco leaves, based on a combination of clean-up procedure: LLE on Chem. Elut columns with diatomaceous earth material and SPE on PestiCarb/NH_2. Meanwhile, plants treated either in spraying or irrigation, we investigated the residual dynamics of pymetrozine in green tobacco leaves using the established analytical method.

EXPERIMENTAL

Chem Elut columns (packed with diatomaceous earth material) and PestiCarb/NH_2 cartridges (500 mg of graphitized carbon black and 500 mg of primary secondary amine) were purchased from Agela Technologies Inc. (USA). Acetonitrile,

ethyl acetate, toluene, *n*-hexane, dichloromethane and acetic acid were supplied by Shanghai Reagent Factory (Shanghai, China). All chemicals were of pro analysis grade. HPLC grade of acetonitrile was obtained from Honeywell Burdick and Jackson (USA). Ultra pure water was obtained from a MILLI-R04 purification system (Millipore, Bedford, PA, USA). All the solvents and samples were filtered through a membrane filter (0.45 μm) before HPLC runs.

Pymetrozine (> 99 %) reference standard was purchased from Ehrenstorfer (Augsburg, Germany). A stock solution of pymetrozine was prepared in acetonitrile at a final concentration of 100 μg mL^{-1} and stored at -20 ºC. This solution was further diluted with 0.020 mol L^{-1} ammonium acetate buffer to obtain HPLC calibration standards at concentrations of 0.010, 0.050, 0.100, 0.500, 1,000 and 5.000 μg mL^{-1}. Each determination was performed in triplicate.

The HPLC system consisted of a gradient pump (LC-10ATVP, Shimadzu, Kyoto, Japan), a manual injector, a Shim-pack VP-ODS column (particle size 4.6 ± 0.3 μm, 150 mm × 4.6 mm i.d., Shimadzu, Kyoto, Japan), a UV variable-wavelength Detector (SPD-10AVP, Shimadzu, Kyoto, Japan) and a data processing system (N2000, Zhejiang University).

Pymetrozine field experiments: A field trial was conducted during the spring season of 2007 on tobacco at the Agricultural Experiment Station, University of Science and Technology of China (USTC), Hefei of China. The experiment field soil is stick disc yellow brown soil. Experiment utilized randomized complete block designs replicated three times and single untreated guard rows separated plots. Every plot has two rows, 7.8 m (15 plants) by 1.2 m (1 row). Except for using pymetrozine, recommended production practices were followed[15]. Tobacco seedlings (K326, flue-cured tobacco) were transplanted into experimental plots on 15th April. Four weeks after transplanted, when tobacco plants were in the rapid growth stage, pymetrozine 25 % WP (a.i, 250 g kg^{-1}, Naming Panfeng Chemical Co., Ltd) was applied to plots at the amounts of 99 g (a.i) ha^{-1}, 6 mg (a.i) $plant^{-1}$, with spraying and irrigation, respectively. Foliar spraying treatment was applied at 50 mL $plant^{-1}$, 0.12 mg mL^{-1} pymetrozine solution, with a hand sprayer (0.7 mm nozzle diameter). Irrigating applied was made with a measuring cup at 200 mL $seeding^{-1}$, 0.03 mg mL^{-1} pymetrozine solution, on the base soil of test plants.

Sampling and sample storage: Five tobacco plants were selected randomly from each plot at 1, 2 and 4 weeks after using pymetrozine. Each time the 10th leaf from bottom to top of the selected plant was picked and then, the five leaves from the different plants were mixed to a sample. These samples of green tobacco leaves were dried at 45 ºC for 10 h. Afterward, each sample's leaves were chopped to pass a 0.5 mm screen and stored in glass jars container at -20 ºC until extraction.

Sample preparation and clean up: Green tobacco leaf samples (5 g, dry weight) were weighted into an Erlenmeyer flask and extracted using 50 mL acetonitrile/water (85:15, v/v) with ultrasonic agitation for 0.5 h. The sample mixture was filtered through a 35 mm Buchner funnel, then the residue cake on the funnel

was washed with 10 mL acetonitrile/water (85:15, v/v). The extract was transferred into 250 mL round flask and concentrated to about 15 mL total volume by a rotary evaporator at 50 ºC under vacuum.

The concentrated aqueous extract was applied to a Chem Elut SPE cartridge. After the liquid has drained into the cartridge, wait for 10 min to obtain an equilibrium distribution in the filling material. The column was eluted with 120 mL ethyl acetate. The eluate was collected in a 500 mL round bottom flask, evaporated under vacuum and then, the remaining solvent was evaporated to dryness under a nitrogen stream.

The residue was dissolved with 10 mL acetonitrile/toluene (3:1, v/v). The solution was applied to a PestiCarb/NH_2 SPE cartridge preconditioned with 20 mL acetonitrile/toluene (3:1, v/v) and eluted with 20 mL acetonitrile/toluene (3:1, v/v). Finally the eluate was evaporated in a rotating vacuum evaporator with a water-bath at 50 ± 1 ºC and then dried under a nitrogen stream. The residue was dissolved in 1 mL acetonitrile/aqueous ammonium acetate (10:90, v/v) and filtered through Teflon filter (0.45 μm) for final HPLC analysis.

Recovery study: Recovery experiments were carried out at three fortification levels and three replicates. Green tobacco leaves as blank samples were planted with the nutrient solution in the greenhouse. Pymetrozine standard solution was added to the green tobacco leaf powder (5 g) to give the samples concentrations of 0.02, 0.2 and 2 μg g^{-1} of pymetrozine. The samples were extracted, cleaned and analyzed following the procedures describe in the section of "Sample preparation and clean up".

Analysis of pymetrozine: The mobile phase consisting of acetonitrile and 0.020 mol L^{-1} ammonium acetate buffer (10:90, v/v), pH 5.0, was sonicated before use for 15 min in an ultrasonic bath to remove air bubbles. The excitation wavelength of the ultraviolet detector was set at 299 nm. The flow-rate was 1.0 mL min^{-1}. The injection volume was 20 μL and the analysis was performed at 30 ºC. Under these conditions, the retention time of pymetrozine was about 11 min. Fig. 2 shows a HPLC/UV chromatogram of a standard solution with 1.00 μg mL^{-1} of pymetrozine under these chromatographic conditions.

RESULTS AND DISCUSSION

Sample extraction and clean up procedures: Tobacco leaves contain more than 4000 chemical components. In order to simplify the process of purification, the extraction should contain a minimum of co-extractives from samples. In this study, selection of extraction solvents is very important in establishing the analytical method. Mastovska and Lehotay[16] has summarized the attributes of the three extraction solvents (acetone, ethyl acetate, acetonitrile) and ordered them according to their suitability for sample preparation in the analysis of pesticide residues in fruit and vegetables as follows: acetonitrile > ethyl acetate >> acetone[16]. 10-20 % water in acetonitrile could improve the extraction efficiency[17]. Therefore, acetonitrile/water was chosen as extraction solvent. Some analysis methods have used acetonitrile/

water for monitoring pesticide residues in tobaccos or foods[18,19]. Further investigation showed that acetonitrile/water (85:15, v/v) with ultrasonic extraction (UE) provided high extraction recovery and relatively low-level matrix interferences.

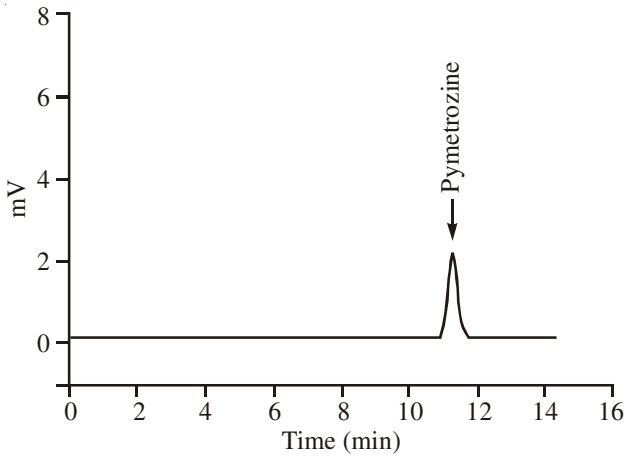

Fig. 2. HPLC/UV chromatographic of a standard solution with 1.00 μg mL^{-1} of pymetrozine

Pesticides in the aqueous sample extract must be transferred into medium-polarity organic solvents such as *n*-hexane or ethyl acetate to remove water and water-soluble co-extractives. Usually, classical LLE or SPE using diatomaceous earth materials has been used in this procedure[20,21]. The Chem Elut column, a well known sample preparation device be used to replace with LLE procedure, was suitable for purifying pesticides residues from aqueous extracts[22].

In order to quantitative pymetrozine LLE was carried out on Chem Elut columns, several eluting solvents such as acetonitrile, ethyl acetate, *n*-hexane and dichloromethane were tested. Practically, 15 mL acetonitrile/water (85:15, v/v) containing 10 ng pymetrozine was applied to the column, which was eluted six times with 20 mL of the tested solvent. The six fractions were collected separately and then the volume of each fraction was evaporated under vacuum to a small volume at a bath temperature of 40 ºC and concentrated to dryness under a slight nitrogen stream at room temperature. The residue was dissolved with 1 mL of the mobile phase and analyzed by HPLC-UV. The elution sounds efficiency of pymetrozine extraction using the four different solvents is shown in Fig. 3. The highest recovery of pymetrozine was obtained after elution with ethyl acetate.

Although the Chem Elut cartridge was used to remove water and water-soluble co-extractives, the eluate of green tobacco sample (5 g) extraction from the Chem Elut SPE cartridge contains the target compound and various hydrophobic co-extractives such as lipids or coloured substances. Fig. 4 shows the chromatograms have interference peaks around the peak of the target analyte, therefore, the eluate should be clean up with other SPE cartridges again.

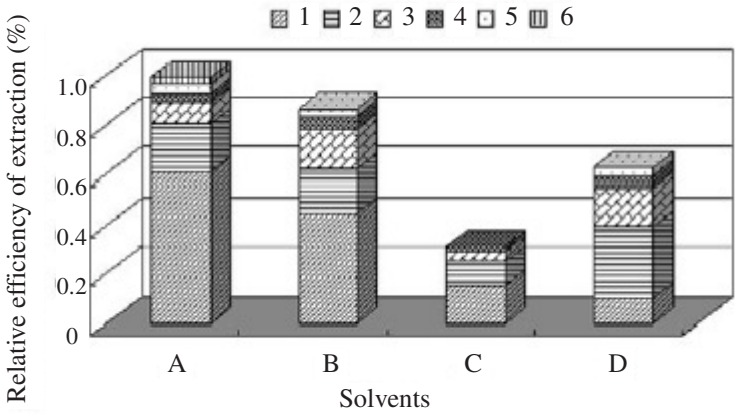

Fig. 3. Relative efficiency of pymetrozine extraction on Chem Elut columns with different solvents. (A) ethyl acetate, (B) dichloromethane, (C) *n*-hexane, (D) acetonitrile. Six fractions were analyzed separately

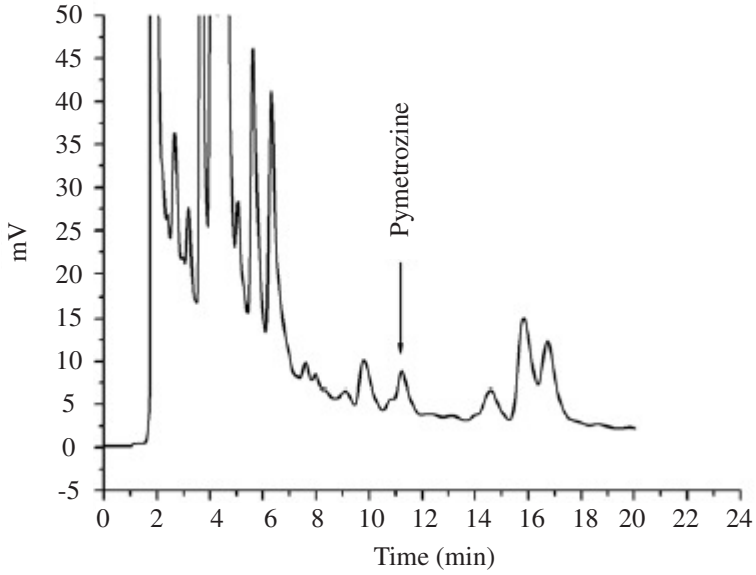

Fig. 4. Chromatogram of sample solution after a Chem Elut SPE cartridge from an extract of a green tobacco sample

Different types of materials have been used for separation of pesticides from co-extractives, in particular, silica, florisil and alumina[23,24]. But some studies which purification tree and vegetable leaves have shown that the efficiency of clean up in different materials is as follows: silica < florisil < florisil + alumina ≅ ENVI™ Carb[25]. In addition, carbon is the only one to give colorless eluates. Furthermore, SPE cartridge (graphitized carbon black) was prominently applied to analyze pesticide residues in tobacco[26]. Thus the use of PestiCarb/NH_2 SPE cartridge (500 mg of

graphitized carbon black and 500 mg of primary secondary amine) has been selected as the second SPE to purify the eluate from the Chem Elut SPE cartridge. Fig. 5 shows a HPLC/UV chromatogram without interference peak around the peak of pymetrozine pesticide.

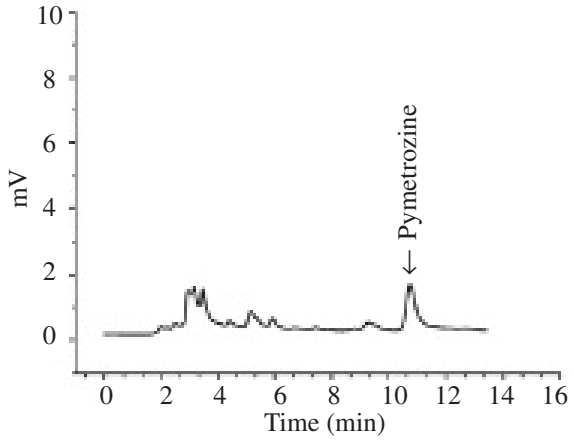

Fig. 5. Chromatogram of sample solution cleaned up with a Chem Elut SPE cartridge and a PestiCarb/NH_2 SPE cartridge from an extract of a green tobacco sample

Calibration curve, limit of detection, reproducibility and recovery: Calibration curves were established by six standards with pymetrozine concentrations ranged from 0.01-5.00 μg mL^{-1}. The selected concentrations of standards covered the expected concentration range of samples. Calibration graph for pymetrozine was obtained by plotting concentration against peak area. The regression between peak area (A) and concentration (C, μg mL^{-1}) yielded the following equation:

$$A = 35069C + 658.25 \ (n = 6, r = 0.9998)$$

The limit of detection (LOD) can be defined as the lowest concentration giving a signal-to-noise ratio (S/N) of 3, is 0.005 μg mL^{-1}. The reproducibility of the method was checked at 0.500 μg mL^{-1} pymetrozine. The relative standard deviation (RSD) of peak area was 2.1 %. The mean recoveries of the method at spiking levels 0.02, 0.2 and 2 μg g^{-1} appears from Table-1. Satisfactory results were found in each instance, with recoveries ranged from 97.69-98.25 %. These results indicate that the method is reliable.

TABLE-1
RECOVERY AND RSD OF PYMETROZINE FROM GREEN TOBACCO LEAVES (n = 3)

Added (μg g^{-1})	Detected (μg g^{-1})			M.R. (%)[a]	RSD (%)[b]
	No. 1	No. 2	No. 3		
0.020	0.0196	0.0193	0.0198	98.12	1.29
0.200	0.1960	0.1950	0.1960	97.69	2.67
2.000	1.9620	1.9740	1.9550	98.25	1.22

a: M.R.: mean recovery. b: RSD: relative standard deviation.

Application to actual samples: Using the method developed, tobacco samples collected at different time from the trial field were analyzed. Fig. 6 illustrates the average concentrations of pymetrozine residues in green tobacco leaves. Under the same amount of pymetrozine was used in either spraying or irrigating treatment for each plant, it can be seen that the average concentration of pymetrozine processed by sprayed is about twice that by irrigated and the spray treatment is more conducive to the absorption and effect of pest control (data unpublished). The data also demonstrates that pymetrozine residue sharp declined from the first week to the second week and changed slowly from the second week to the fourth week in the two treatments. In addition, the residue levels of the two treatments were very low after four weeks. The amount of pymetrozine in test obey to the recommended doses in tobacco production[27]. The results show that the residue data of the samples are far less than GRLs (1.00 μg g^{-1}) established by the COREST under this application, thus it can be accepted by the tobacco industry.

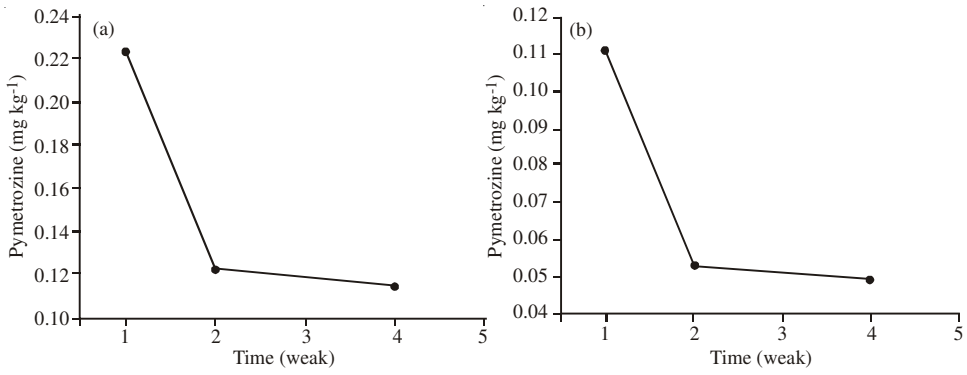

Fig. 6. Concentrations of pymetrozine residues in green tobacco leaves by disposed of two different modes at different times: (a) spraying, (b) irrigation

Conclusion

An analytical method for the determination of pymetrozine residues in green tobacco leaves has been developed. The proposed method involves extraction with acetonitrile/water, clean-up with two SPE cartridges (a Chem Elut SPE cartridge and a PestiCarb/NH_2 SPE cartridge) and analysis by HPLC/UV. The highest recovery, low LOD and best repeatability, ensured that the method was reliable and can be utilized for regular monitoring of pymetrozine residues in green tobacco leaves. The residues of field trials confirmed that the surface spray is in favour of the absorption of pymetrozine. Higher residues of the active ingredients in tobacco plants were appeared in 1-2 weeks after use of pymetrozine. Under the recommended dosage, the residues are much lower than the guidance residue limits.

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