Chapter 7

Determination of Paclobutrazol Residue: Method Validation of PBZ Residue in Soil

7.1 Abstract

The application of solid-phase microextraction (SPME) for soil sample in paclobutrazol residue analysis was validated. This technique coupled mainly to gas chromatography with mass spectrometry (GC-MS). The preparative soil procedures for transportation, extraction of paclobutrazol, extracted time and analysis were obtained. Recoveries for paclobutrazol from spiked soil samples at 0.7, 0.5, 0.1, 0.05, 0.01, and 0.005 milligrams per kilogram were averaged 67 %. Limit of detection was found at the concentration of 0.01 milligram per kilogram sample, the extraction time was at 30 minutes and the paclobutrazol under sterile condition was highly stabilized. Thus, the method validation for determination of paclobutrazol in soil is suitable, the simple dilution of the extraction to avoid negative matrix effects is more important.

7.2 Introduction

Bhattacherjee and Singh (2002) used gas chromatographic for the estimation of paclobutrazol residues in soil. Methanol was used for extraction of paclobutrazol from soil samples. Electron capture detector (ECD) was chosen for gas chromatographic analysis. Singh and Bhattacherjee (2005) studied the undertaking chemical residue in soil for analyzing the persistence of paclobutrazol by GLC method. Methanol was used to extract paclobutrazol residues in soil samples. Bolygo and Atreya (1991) developed a multi-residue analytical method for the analysis of paclobutrazol in groundwater by GLC using nitrogen–phosphorus detector (NPD). HPLC with UV detector was used analysis paclobutrazol in soil (Subhadrabandhu *et al*., 1999). The main objective of this work is to describe a method validation, proposed for analysis of paclobutrazol residues in soil using a sample preparation step by conventional extraction coupling with SPME extraction and analysis by GC-MS.

7.3 Materials and Methods

Materials

Mixer-Grinder (diameter of metal beads 3 cm, Retsch, Haan, Germany)
Autoclave (Hiclave Hv-85, Hirayama, Tokyo, Japan),
(Type FVS3, Fedegari, Italian)
Sieve machine (5 sieves mesh size: 3.15 mm- 1mm- 0.5 mm- 0.315 mm- 0.25 mm, Retsch, Haan, Germany)
Sieve (Stainless steel, diameter 8 inches, and 3.35 millimeter mesh size, Endecotts Ltd, London, England)

7.3.1 The extraction of paclobutrazol residue in soil

GC-MS Conditions

The GC-MS conditions was followed in Chapter 5

SPME procedure

The SPME conditions and processes were followed in Chapter 5

Preparation of spiking soil sample for recovery studies, detection limit, and sterile analysis

The soil samples have been collected at three depth levels from University of Hohenheim and Mae Jo University field for spiking and sterile experiment. Soils were frozen (- 20 ˚C) and cut into 3 segments according to the corresponding depth (0-5, 5-10, and 10-20 centimetres). Samples were dried at 105 ˚C for 1 hour and cooled down. Stones were removed 2 times and 10 minutes at a time by sieving machine (5 sieves mesh size: 3.15mm- 1mm- 0.5mm- 0.315mm- 0.25mm, Retsch, Haan, Germany) at 70 % of speeds. Then, soil samples were weighed exactly 100 grams (± 0.001) into a glass bottle, the paclobutrazol standard 0.7 milligrams per kilogram sample, distilled water with a ratio of 1:1 (w/w, water : dry soil), were added. The mixer was continuously shaken for 24 hours. Next, these were taken arid again at 100 ˚C for 3 hours and milled by Mixer-Grinder (diameter of metal beads 3 ccentimetres, 5 beads Retsch, Haan, Germany) for 5 minutes at 60 % of speeds. The fine soil samples were kept in the pouches for sterilization and spiking analysis. As shown in Figure 7.1.

Preparation of soil samples for transportation

The soil samples have been collected at three depth levels from the target areas or the ring segments used for paclobutrazol application around each tree (see appendix). Only one sample was chosen from 12 sampling segments in each tree. Five trees were investigated. The cylinder of soil samples were frozen, cut into 3 segments according to the corresponding depth of the soil layer on the field and weighed. The soil samples were dried at 105 ˚C for 1 hour, cooled down and weighed. Stones of the dried soil samples were removed by sieving at a mesh size of 3.35 millimetres and weighed. Then, it was transferred into the glass bottles and weighed again. For transporting to University Hohenheim, the sieved soil was sterilised at 121 ˚C for 20

minutes (Hiclave, HV-85) in mode of liquid. After that it was dried again at 105 ˚C for 30-45 minutes, cooled down and weighed. Finally, it was filled in pouches and sealed which was ready to transport to Germany. As shown in Figure 7.2.

The extraction procedure of paclobutrazol from soil samples

A soil sample after milling of 100 grams was exactly weighted (± 0.001) into a 500 milliliters of glass bottle, the internal standard (diclobutrazol [DBZ] 0.7 milligrams per kilogram sample), and magnetic stir bar was added and homogenized for 5 minutes. The distilled water was added in the ratio of 1:1 (w/w) and homogenized as well as by stirring for 5 minutes. Acetonitrile and water (Crook, 1999) in ratio of 70.0 %:30.0 % (w/w); respectively, were added and the mixture was homogenized for 30 minutes using magnetic stirring at high speed. The 20 grams of Celite 503 was added to supportable soil during the filtration and stirred for 2 minutes. The extraction bottle was weighed and tare. The mixture was filtered through a 110 millimeters Büchner funnel with paper filter (No.589/3, Blue ribbon, Microscience GmbH, Dassel, Germany) associated with vacuum to the flask, and the mixture used was weighed for calculation. The bottle was washed 4 times with 5 grams solution (ACN/H_2O, 70.0 %:30.0 %, w/w), rinsing of filter cake 2 times with 5 grams solution (ACN/H_2O, 70.0 %:30.0 %, w/w) and weighed again. The mixture solution was evaporated under the vacuum (30 ˚C, <150 millibars) but not until dryness, only until ACN has been removed 69.7 % weight loss. The addition of acetonitrile was used to adjust the ACN/H_2O ratio (3.5 %:96.5 %, w/w). Finally, the supernatant liquid 1 milliliter was collected and diluted to 10 milliliters (ACN/H_20, 3.5 %:96.5 %, w/w) in volumetric flask with additional weight control. It was filtered through a 70 millimeters glass funnel with paper filter (No.595½, Microscience GmbH, Dassel, Germany) to glass bottle. Every step just referred to the MS-excel® sheet form for the calculation (FB), see appendix. The scheme of the extraction was shown in Figure 7.3.

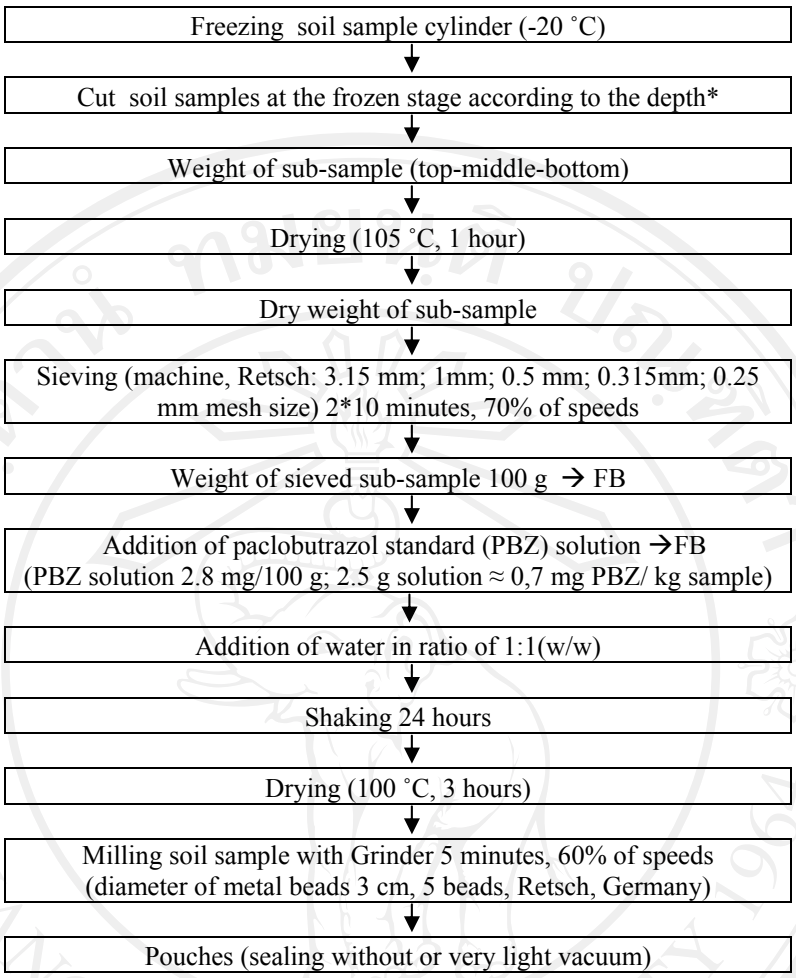
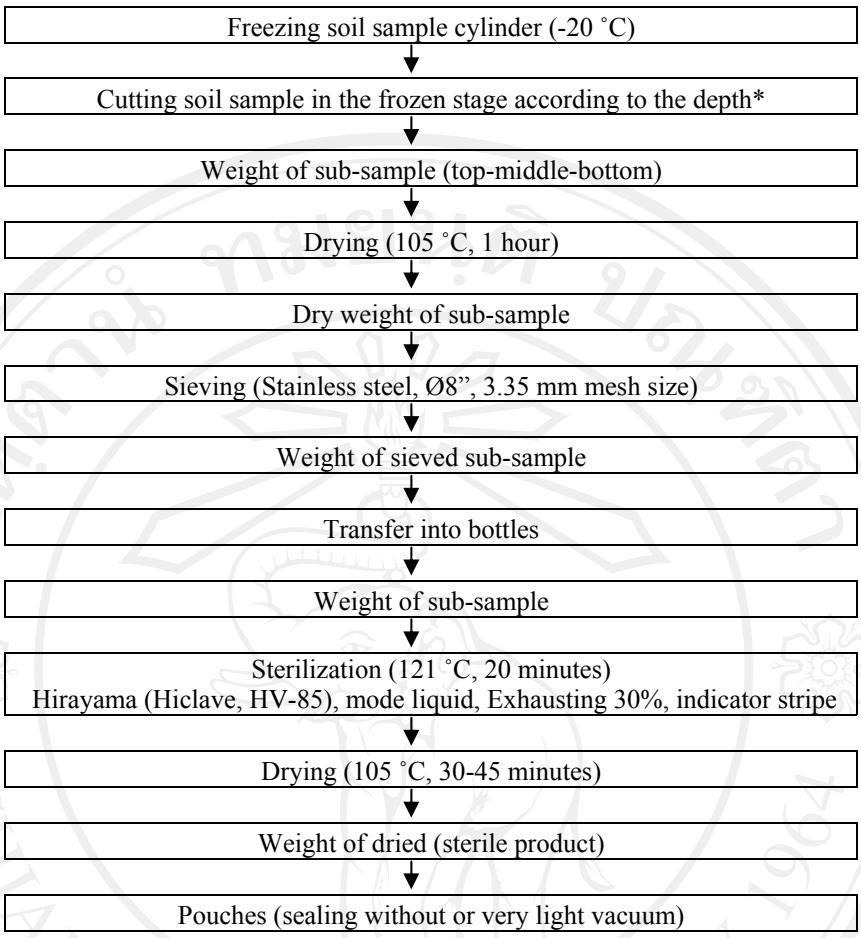


Figure 7.1 Scheme of spiking paclobutrazol for recovery and detection limit analysis (Reintjes, 2005).



* Top = 0 - 5 cm, Middle = 5 - 10 cm and Bottom = 10 - 20 cm

Figure 7.2 Soil samples procedure for transportation (Reintjes, 2005).

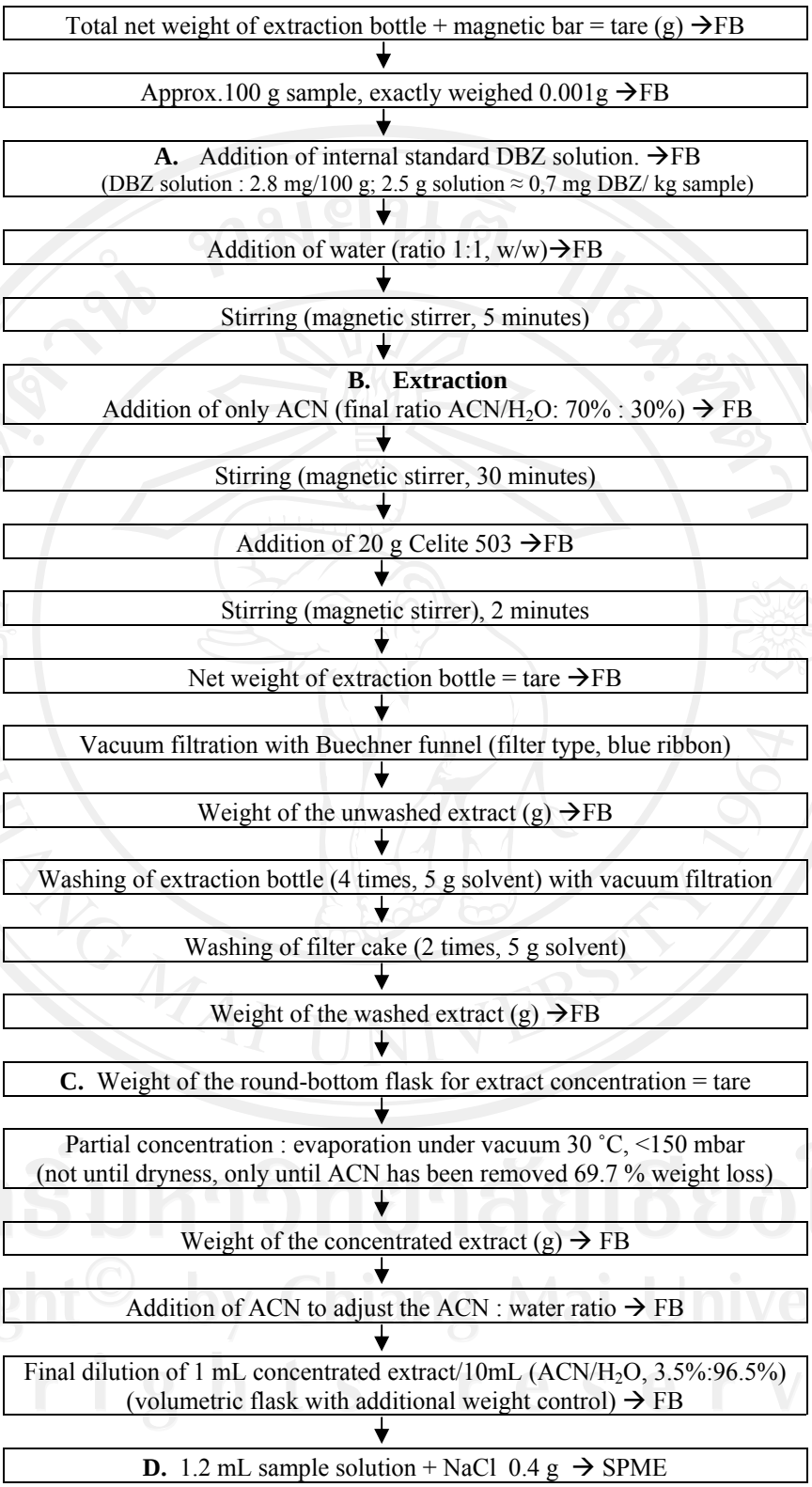


Figure 7.3 Scheme of soil samples extraction procedure. A. addition of internal standard, B. sample extraction, C. concentration step, D. SPME extraction.

7.3.2 Method validation

Stability of paclobutrazol in soil under the sterile condition

The stability of paclobutrazol under sterilization condition, 121 ˚C at 20 minutes by Autoclave (Type FVS3, Fedegari, Italian) in Institute of Microbiology (150a), University of Hohenheim, was studied by weighting 100 grams (± 0.001) soil samples were and preparing as previously described (Figure 7.1). Then it was followed as the same as the scheme of soil extraction procedure. The data were compared with control (no sterilized).

Optimization of paclobutrazol extracts time in extraction soil procedure

A 100 grams of each sample was taken (previously described, PBZ 0.7 milligrams per kilogram sample added) and then the internal standard (diclobutrazol [DBZ] 0.7 milligrams per kilogram sample) was added and homogenized. Samples were extracted by acetonitrile (ACN) and water (70.0 %:30.0 %, w/w). The homogenization was done by stirring for various times of 10, 30, and 60 minutes, with the addition of Celite 503 and filtrating. The mixture solution was evaporated under the vacuum (30 ˚C, <150 millibars) but not until dryness, only until ACN has been removed 69.7 % weight loss. The supernatant liquid (1 milliliter) was collected and diluted to 10 milliliters (ACN/H_20 3.5 %:96.5 %, w/w) as above described (Figure 7.3.) After the dilution, 1.2 milliliters of sample was transferred to a vial containing 0.4 grams sodium chloride. Vials were shaken or stirred by magnetic until complete dissolution occurred. The samples were then analyzed using GC-MS in the electron-impact ionization (EI) mode.

Recovery study of paclobutrazol in the soil

The soil samples have been collected at three depth levels from Mae Jo University field for spiking and recovery study of paclobutrazol in soil samples. Soil samples were prepared as shown as in Figure 7.1. Then the extraction was followed

according to Figure 7.3. After the dilution, 1.2 milliliters of sample was transferred to a vial containing 0.4 grams sodium chloride. Vials were shaken or stirred by magnetic until complete dissolution occurred. The samples were then analyzed using GC-MS in the electron-impact ionization (EI) mode.

Detection limit and recovery study of paclobutrazol in the soil

The pooled soil samples were used for this experiment. Samples were prepared as described in Figure 7.1; the only difference was the concentration of paclobutrazol which was added at 0.005, 0.01, 0.05, 0.1, 0.5 milligrams per kilogram. The extraction of paclobutrazol was processed as in Figure 7.3. The samples were then analyzed using GC-MS in the electron-impact ionization (EI) mode.

7.4 Results

7.4.1 Method validation

Stability of paclobutrazol under the sterilization condition

Stability of paclobutrazol under the sterilized condition of 121 ˚C for 20 minutes was observed. The treatments in comparison to control (not sterilized) were not significantly different in terms of volume of the concentrations and recovery percentage (Figure 7.4). The recovery of paclobutrazol in S2 treatment was below the theoretical line but the mean of total was raised. In other words, paclobutrazol was highly stable, although it was subjected to high pressure and temperature at the same time. Thus, the preparation of soil for transportation had to meet the restrictions of European's laws. Soil had to be eliminated for contamination of microorganism in the sample before shipment.

Optimizations of paclobutrazol extract time in soil extraction procedure

The various extract times of 10, 30 and 60 minutes were evaluated in soil extraction procedure. The recovery percentage of paclobutrazol at 10 and 60 minutes were below the theoretical concentration line of paclobutrazol were 72.9 % and 71.5 %, respectively. However, 30 minutes extract time in which the recovery was 100.9 % was better than the others as shown in Figure 7.5.

Recovery of paclobutrazol in the soil

The results of the recovery studies are given in Table 7.1. The percent mean recovery was below 78 % from all depth levels. The recoveries from each level as top (0-5 cm), middle (5-10 cm) and bottom (10-20 cm) layers were observed to be 64.56 %, 77.75 % and 50.78 %, respectively. However, the standard deviation (S.D.) and coefficient of variant (CV) in all cases were achieved in range of 3-11 %. When the recovery in Table 7.2 was inclusively calculated, the mean was about 67 %.

Detection limit of paclobutrazol in the soil

The limit of detection was obtained at 0.01 milligram per kilogram sample, although the recovery was beneath 55.80 %. The peak of paclobutrazol at the concentration of 0.005 milligrams per kilogram was not found in the present case. The detectable means of paclobutrazol were also lower than the expectation. The standard deviation (S.D.) and coefficient of variant (CV) were satisfactory. All of mean recovery values were at an acceptable level at percentage coefficient of variant (CV) values of $\leq$ 20 %. In the experiment at the concentration of 0.05 milligrams per kilogram, it gave approximately 16 %. The quantitative analysis of the linearity of response of the concentration is displayed in Figure 7.6. Linearity plots obtained indicated a high correlation coefficient values ($R^2 > 0.99$) of the linear regression analysis, showing for fitting the paclobutrazol concentration rate in spiking soil.

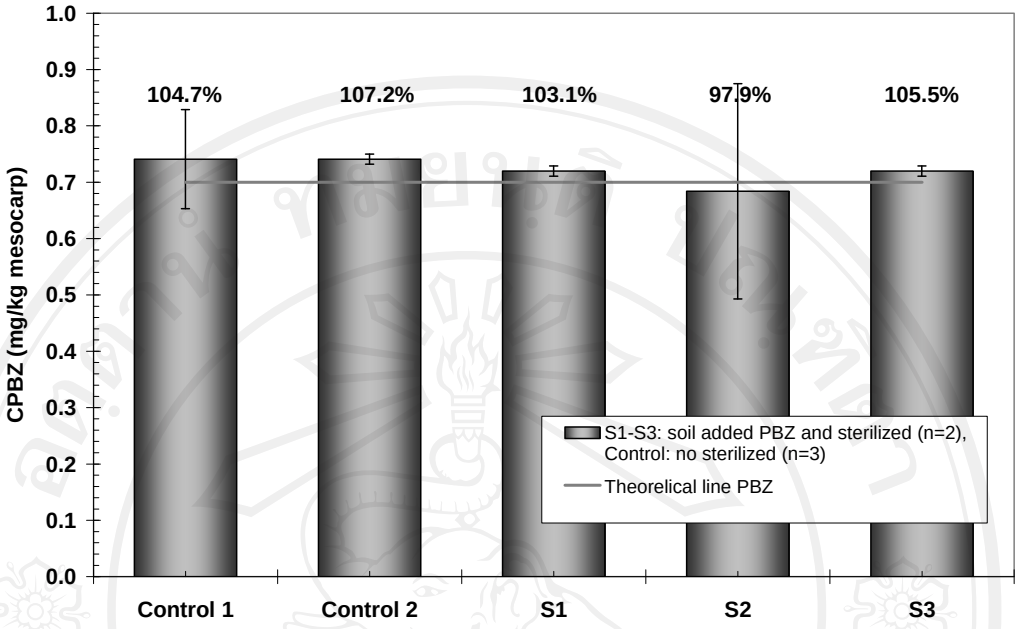


Figure 7.4 Stability of paclobutrazol in pooled soil samples under sterilized condition 121 ˚C, 20 minutes (adapted from Reintjes, 2005).

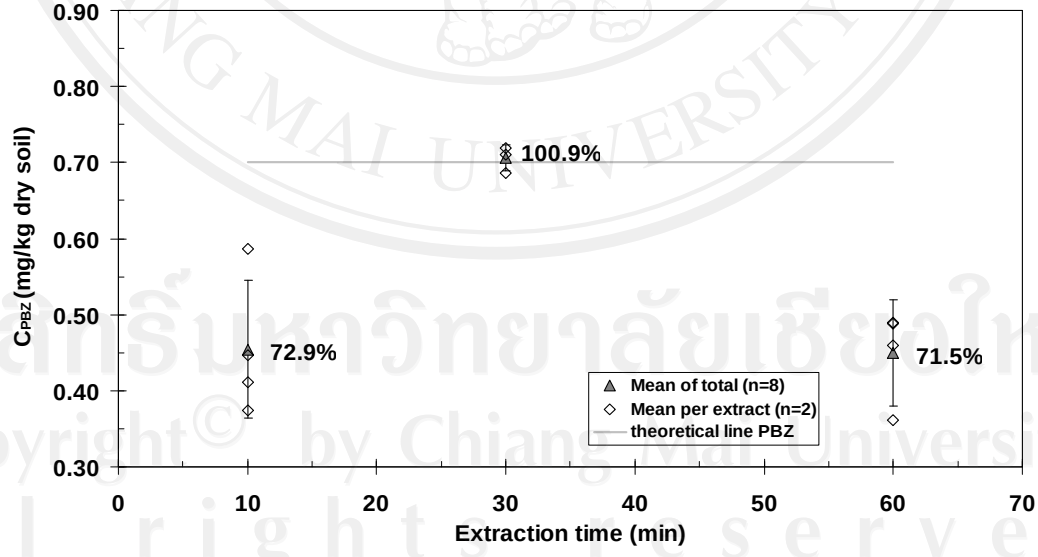


Figure 7.5 Recovery percentage of optimizing extract time in soil extraction procedure (adapted from Reintjes, 2005).

Table 7.1 Recovery of paclobutrazol from fortified processes in different soil depths (Reintjes, 2005).

Soil depth (cm)	Dilute Ratio	Mean Recovery (%)	S.D. Recovery	n	PBZ (mg/kg soil)		
					Expected	Mean detected	CV (%)
0-5	1:10	64.56	7.05	4	0.7020	0.4532	3.94
5-10	1:10	77.75	3.07	4	0.7054	0.5485	8.18
10-20	1:10	50.78	4.15	6	0.7014	0.3562	10.92

Table 7.2 Limit of detection and recovery in different concentration of paclobutrazol in pooled soil samples (Mean data, n=4; ND = not detected) (Reintjes, 2005)

Spiked (mg/kg)	Dilute Ratio	PBZ (mg/kg soil)				Mean Recovery (%)
		Expected Results	Mean Detected	S.D. Detected	CV (%)	
0.5	1:10	0.4909	0.3446	0.027	7.77	70.19
0.1	1:10	0.1025	0.0852	0.010	11.37	83.17
0.05	1:10	0.0524	0.0357	0.006	16.11	69.76
0.01	1:10	0.0137	0.0076	0.000	5.69	55.80
0.005	1:10	0.0052	ND	ND	ND	ND

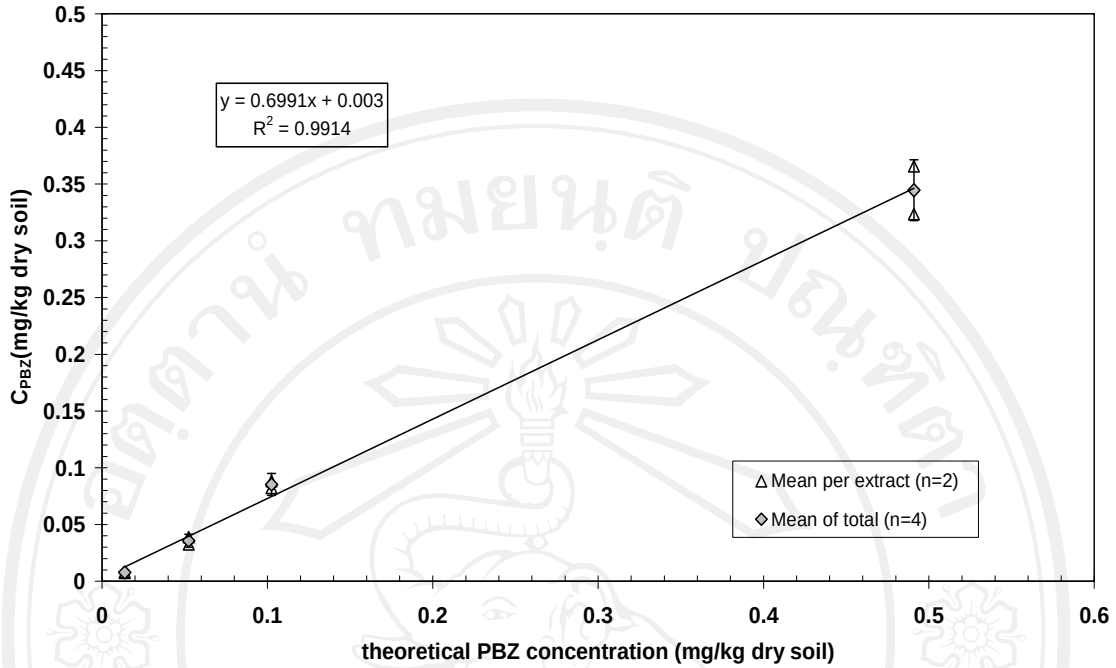


Figure 7.6 Linearity plot by means of the paclobutrazol standard addition method (adapted from Reintjes, 2005).

7.5 Discussion

Stability of paclobutrazol under high temperature and pressure for 20 minutes was confirmed that it was very strong persistency in the environment. The optimum extracts time in the extraction soil procedure was occurred at 30 minutes. It was different from the extraction of mango. It may be due to numerous organic matters of soil components which were bound to paclobutrazol, rather moderately to strongly, were sorbed to soil (Bromilow *et al*., 1999). The extractable fraction may also be sorbed to the soil solid phases (Gevao *et al*., 2000) which increased time or intensity of extraction. Bromilow *et al*. (1999) reported that several triazole fungicides are very persistent, though no deleterious effects on soil microbial processes have been reported. triadimenol, flutriafol and epoxiconazole were all very persistent with DT_{50}>400 days whilst propiconazole had DT_{50} *c* 200 days.

The recovery of paclobutrazol was in medium level (Mean of all were about 67%, Table 7.1, 7.2), could be accepted. On the other hand, Sharma and Awasthi (2005) reported that the percent recovery of paclobutrazol residues was 83±3.4% for soil samples with the syringe injection determined by gas liquid chromatography. In addition, Bhattacherjee and Singh (2002) reported that a simple gas chromatographic method for the estimation of paclobutrazol residues in soil was standardized using electron capture detector and megabore column. Paclobutrazol was to be derivatized as paclobutrazol-TMSi (trimethylsilyl ether) before analysis (Wang *et al*., 1986) that it was not required for GLC-ECD determination of paclobutrazol. Soils were simply extracted in methanol, filtered twice, methanol was evaporated completely and residues were redissolved in 1 milliliter before analysis. An average recovery of 86.82 % of paclobutrazol was obtained from soils fortified with 0.1, 1.0 and 2.0 ppm solutions with a minimum detection limit 0.0001 ppm. Also Shalini and Sharma (2006) found that the percent mean recovery of paclobutrazol residues from soil at fortification levels of 0.01 and 0.1 microgram per gram was 85.4 % and 89.2 %. Beltran *et al*. (2000) mentioned that the organic matter in the soil sample greatly influenced the recovery of compounds from the soil. The resultant paclobutrazol recovery in different deep soils were not excellent, it may be influenced by various factors such as degree of its adsorption on soil particles, rate of its release from soil particles to soil solution (Jacyna, and Dodds, 1995). Including the reason of bound chemical residues in soils as previously described. Moreover, the quantitative application of SPME to soil samples does not allow the direct use of external standard calibration curves, being necessary to use internal standard quantitation or the standard addition procedure (Beltran *et al*., 2000).

Limit of detection was also achieved as 0.01 milligram per kilogram, while the peak area in the concentration of 0.005 milligrams per kilogram was not found. However, if the supernatant was diluted in grater dilutions such as 1:100, 1:1000 or 1:5000, etc., it could probably help (Yang *et al*., 2006). Moreover, to reduce organic solvent content when a previous solvent extraction is required, it is usually achieved by diluting sample extracts prior to SPME application (Beltran *et al*., 2000). In addition, Simplício and Boas (1999) showed that the pesticide recoveries could be

much improved by diluting the samples up to a 100-fold dilution in the determination of organophosphorus pesticides in pear fruit and juice. Nevertheless, in this case it was not necessary know the limit detection at lowest concentration of paclobutrazol in soil as the same to mango pulp.

According to Prosen and Zupančič-Kralj (1998) it indicated that for isolation of residue pesticides and their degradation products solid-phase microextraction (SPME) could be used in combination with conventional extraction method. This modern separation method was optimized for extraction of organochlorine and triazine pesticides from soil samples. Analytes were desorbed from the fiber in the injector of gas chromatograph and determined by either electron capture or mass spectrometric detection. Linearity and limit of detection were tested in the 0.1 – 20.0 nanograms per gram range for organochlorines and 10 – 100 nanograms per gram range for triazines. The method presented could be used for screening of pesticides in contaminated soil samples and offers a simple alternative to established methods of pesticide analysis in soil. Hence, it could be used in similar work and modified for other pesticide analysis in the future.

7.6 Conclusion

In conclusion, this method was veritably suitable for residue analysis of paclobutrazol in soils. However, the dilution was more important or necessary for soil analysis which the technique of SPME had been applied to analyze soil extracts.