Chapter 6

Determination of Paclobutrazol Residue: Method Validation of PBZ Residue in Mango Fruits

6.1 Abstract

The method for the determination of paclobutrazol in mango pulp was developed and validated. The samples were extracted in two steps, conventional method associated with solid-phase microextraction and analysed by gas chromatography-mass spectrometry (GC-MS) using an electron-impact ionization (EI) detector. Limitation of detection was that residue obtained by this method for mango pulp was lower than the maximum levels established by FAO and European legislations. Recovery tests were performed for concentration between 0.05 and 0.5 milligrams per kilogram. Mean recoveries for paclobutrazol were all above 85.0 %. Three mango cultivars (Kent, Haden and Palmer) were selected for determining the effect of cultivars on the recovery percentage of paclobutrazol. For the influence of ripening stage, the cultivar Tommy Atkins was chosen. Prior to the analysis of PBZ, all mango samples were blended with the standard solution of PBZ at the concentration of 0.7 milligrams per kilogram mango. It was found that among the three cultivars studied the recovery of PBZ in cultivar Palmer was the lowest (62.81%). The recovery percentage of PBZ was also affected by ripening stage. It was significantly decreased at day 4 of ripening where the mangoes reached their eating-ripe stage. It dropped from 96.2% at the day of harvest to 83.7% on day 4 of ripening. The extraction of PBZ appeared to be enhanced when mangoes became soft. It was found that the stability of SPME fiber could be prolonged up to 80 times of the injections. Thus, the method has been successfully used for the detection and quantification of paclobutrazol in mango fruits.

6.2 Introduction

The methods of analyzing paclobutrazol have been generally referred by the used of high performance liquid chromatography (HPLC), together with UV detection and different cleanup steps for the separation of paclobutrazol in mango (Chuanfan and Jiuxue, 1998; Subhadrabandhu *et al*., 1999) pear pulps and apple (Bicchi *et al*., 2001). LC, liquid chromatography, has been applied for polar, thermolabile, or low volatility compounds with direct injection of raw extracts. LC-MS, liquid chromatography-mass spectrometry, technique has been also applied for residue analysis of polar pesticides in vegetables (Schwedler *et al*., 2000). However, little work has been published on GC-MS, gas chromatography-mass spectrometry, for the separation paclobutrazol in mango, and those available were not standardized.

Recently, solid-phase microextraction (SPME), an attractive alternative to most of the conventional sampling techniques, has gained widespread acceptance and is advantageously used in many analytical procedure (Meurer *et al*., 2002; Kurtz *et al*., 2003). SPME has become popular in GC analyses since it integrates sampling, extraction, concentration and sample introduction procedures into an easy, rapid, sensitive, inexpensive and single solvent-free step (Pawliszyn, 1999; Meurer *et al*., 2002; Lambropoulou and Albanis, 2003). SPME is normally coupled with GC(/MS), however a simpler coupling of SPME to MS via a short GC transfer line has recently been described to achieve higher sample throughput and to obtain chemical 'fingerprint' characterization of complex mixture (Meurer *et al*., 2002). GC is the most widely adopted technique in pesticide residue analysis because it separates well, is fast and has many available selective and sensitive detectors (Hwang and Lee, 2000). MS is a very powerful tool for the identification and quantification of organic compounds in complex samples (Arrebola *et al*., 2003; Goncalves and Alpendurada, 2004). The main objective of this work is to describe a method validation, proposed for analysis of paclobutrazol residues in mango fruit, especially in mango cultivar 'Tommy Atkins' using a sample preparation step by conventional extraction coupling with SPME extraction and analysis by GC-MS.

6.3 Materials and Methods

Mango Samples for residue analysis was preparerd at Chiang Mai University Since the instrument for determining paclobutrazol residue, the gas chromatography-mass spectrometry (GC-MS) is not available at Faculty of Agricultural, Chiang Mai University these were done at University of Hohenheim

Chemicals and reagents

Paclobutrazol (PBZ 99.5%), Diclobutrazol (DBZ 98.4 %), analytical standards of purity, were purchased from Sigma-Aldrich GmbH, Germany.

Sodium chloride (analytical grade, Merck, Germany), Acetonitrile (isocratic grade for LC 99.8%, VWR, Darmstadt, Germany) Celite 503 (silica; Carl Roth GmbH & Co., Karlsruhe, Germany) were ordered from listed suppliers. Helium (99.999%) was used as the carrier gas.

Materials

Filter paper 2 types:

Filter paper for Buechner funnel diameter size 110 millimeters (mm) No.589/3 (Blue ribbon, Microscience GmbH, Dassel, Germany)

Filter paper for glass funnel diameter size 70 millimeters (mm) No.595½ (Microscience GmbH, Dassel, Germany)

Rotary evaporator (Büchi, rotavapor-R)

Blender (Grindomix GM 200, Retsch, Haan, Germany)

Water bath with water circulator associated with magnetic stirrer (IKA-Combimag, RCO)

Refractometer (Sartorius, Germany)

Infrared-moisture content machine (Sartorius MA 40, Sartorius AG Göttingen, Germany)

6.3.1 Preliminary extraction of paclobutrazol in mango fruit

GC-MS Conditions (alteration from Crook, 1999)

The GC analyses were performed with a Varian model Star 3400 gas chromatography with electronic flow control (EFC) and fitted with Saturn II ion-trap mass spectrometer (Varian Instruments, USA). The mass spectrometer was operated in the electron impact (EI) ionization scan mode. In the scanned mass rang was *m/z* 50-300 with a scan rate of 1000 milliseconds, and segment acquire time 25 minutes.

The GC column used was 30 meters of a BPX5 with a 0.25 millimeters internal diameter and a 0.25 micrometers film thickness, which was inserted directly into the ion source of the mass spectrometer. The GC column temperature program used was to hold the temperature initially at 60 ˚C for 6 minutes (for SPME desorption time). The column was the programmed at a rate of 20 ˚C per minute to a final temperature of 280 ˚C. The column was then held at this temperature for 8 minutes. A column head pressure of 11 p.s.i., injector constant temperature of 300 ˚C and detector constant temperature of 300 ˚C were used. Helium (99.999 %) was used as carrier gas. Samples were manually injected into SPI/1077 splitless program.

SPME procedure (alteration from Crook, 1999)

The polyacrylate (PA) fiber was conditioned before initial application in the hot port (split) of the gas chromatography by heating at 300 ˚C for 3 hours according to the instruction provided by the supplier; this treatment removed the impurities present in the coating and introduced during the manufacture of the fiber.

SPME parameters: 85 micrometer polyacylate fiber (white) for manual holder from Supelco, USA.
Equilibrium time: 45 minutes with constant agitation.
Desorption time: 6 minutes
Desorption temperature: 300 ˚C

All SPME was made directly from the aqueous sample. The vials were filled with 1.2 milliliters aqueous sample or standard solution and with the addition of 0.4 grams sodium chloride. First the fiber was exposed to the stirred sample for an optimized absorption time 45 minutes at room temperature (20 ˚C) and then it was removed from the sample and introduced into the GC injector where the thermal desorption of the analyte at 300 ˚C for 6 minutes was carried out.

Preliminary extracts paclobutrazol in mango pulp

The 100 grams of the mango sample was finely cut and frozen. An aliquot of 10 grams was exactly weighed and added with 25 milliliters of acetonitrile and water with a ratio of 35:65 v/v (8.75 milliliters: 16.25 milliliters, 25 milliliters) in a glass test tube. After that 0.860 milliliters of paclobutrazol stock solution (15.4 micrograms per milliliter) and 0.808 milliliters of diclobutrazol stock solution (16.4 micrograms per milliliter, internal standard) were added in the test tube and mixed well. Sample was then homogenized using ultraturax homogenizer. Sample was then centrifuged at a speed of 4,000 rpm for 5 minutes to obtained separation of the supernatant liquid from the particulate material. This stage contained a high percentage of organic solvent. Therefore, elimination of the solvent affect was crucial. The 0.25 grams aliquots of the supernatant liquid were taken and diluted to a final volume of 10.0 milliliters to give a final sample concentration of 0.01 microgram per milliliter. After the dilution, 1.2 milliliters of sample was transferred to a vial containing 0.4 grams sodium chloride. These vials were thoroughly mixed by magnetic stirrer until completely dissolved. Sample was analyzed using GC-MS in the EI mode and compared with standard solution in water.

6.3.2 Optimal GC-MS condition

Instrumentation

The GC-MS analysis was performed by a Varian model Star 3400 gas chromatograph equipped with Electronic Flow Control (EFC) and fitted with a Saturn II ion trap mass spectrometer (Varian Instruments, Walnut Creek, CA, USA).

The GC chromatographic column consisted of a BPX5 capillary column (SGE GmbH, Darmstadt, Germany), length 30 meters, internal diameter (I.D.) 0.32 millimeters and containing 5% phenyl-polysilphenylen-siloxane with a phase thickness of 0.5 micrometers connected to the splitless injector (The I.D. and thickness were changed from the previous test (I.D.=0.25 millimeters, thickness = 0.25 micrometers). The carrier gas was helium (99.999%).

Gas chromatographic (GC) and mass spectrometry (MS) detection condition *(Alteration from Crook, 1999)*

The oven temperature program of GC was to hold the temperature initially at 60 ˚C for 6 minutes to a final temperature of 280 ˚C at a rate of 20 ˚C per minute and then held at this temperature (280 ˚C) for 8 minutes. A column head pressure of 11 p.s.i. and an injector temperature of 300 ˚C were used. The injector was operated by manual holder into splitless mode (SPI/1077) for 6 minutes, the lapse of time for SPME fiber desorption and set at a fixed constant temperature of 300 ˚C. The GC transfer line was maintained at continual 300 ˚C. The mass spectrometer was operated in the electron-impact ionization (EI) scan mode with a source temperature of 300 ˚C. Ionization mode was obtained at fixed mode. The electron energy was 70 eV and the filament current 10 μA. The manifold temperature was set at 180 ˚C. The electron multiplier voltage was established at 1800 volts. The amplitude voltage (A/M) was 4.0 volts. The external event 1 was turned on.

Chromatograms were acquired in ‘scan’ mode scanning the mass range from *m/z* 50 to *m/z* 300 (with a scan rate 1000 milliseconds), with a back ground mass of

m/z 45 segment acquire time 25 minutes. In order to improve the peak identification, three fragment ions were monitored from the spectrum of each compound to quantify the response in the selected-ion monitoring (SIM) mode. The mass spectrum of *m/z* 125, 236 and 294 for paclobutrazol (Retention time 17.038 ± 0.2 minutes) and *m/z* 159, 270 and 272 for diclobutrazol (Internal standard, Retention time 17.518 ± 0.2 minutes) were ion monitored as references. In this way it could be easily identified.

Solid-phase microextraction fiber (SPME)

The SPME holder and fiber assembly for manual sampling were provided by Supelco (Bellefonte, PA, USA) and used without modification. The silica fiber coated with an 85 micrometers (μm) thick polyacrylate (PA) film. Prior to the measurements, the fiber was conditioned in the injector for 3 hours at 300 ˚C, with the split vent opened, to fully remove any contaminant or impurities present in the coating and introduced during the manufacture of the fiber which might have cause very high baseline noise and injected into the GC system until interfering peaks disappeared. This was according to instruction provided by the supplier.

Solid-phase microextraction (SPME) analytical procedure
(Alteration from Crook, 1999)

All SPME was made directly from an aqueous sample. The vials were filled with 1.2 milliliters aqueous sample or standard solution with the addition of 0.4 grams sodium chloride (NaCl, Merck). The vials were then sealed with the hole caps and Teflon-faced silicone septa (Supelco, USA). The samples were stirred for dissolving the salt at 15 minutes. Then, the vials were placed in water bath which circulating water associated with magnetic stirrer (IKA-Combimag RCO.) that controls the temperature at 35 ˚C, with stirring again to adjust the temperature of aqueous sample to 35 ˚C at 15 minutes. The speed of stirring was set at level 3.5. Next, the fiber was then exposed to aqueous phase for an appropriate time period of 60 minutes, with stirring sample at the control temperature 35 ˚C (in order to improve mass transfer from the aqueous sample into the fiber coating). After extraction, the fiber was

removed from the sample and directly introduced into the hot injector of the GC system for analysis (desorption). Where the thermal desorption of the analysis at 280 ˚C for 6 minutes was carried out. After the desorption, the fibers were reused but they should be inserted into the split port for 10 minutes at 300 ˚C and turned on the helium (He) gas flowing at 11 p.s.i. for cleaning proposes. Then, the next sample could be continually operated.

Optimal Absorption time, desorption time and clean up time of SPME fiber

The optimization of adsorption time, desorption time and cleaning up time of SPME fiber were studied in the Chapter 5. They were observed at 60, 6, and 10 minutes, respectively. This data were used as following for investigation of paclobutrazol in mango fruit analysis.

Preparation of standard solutions

To prepare individual stock standard solutions (paclobutrazol, PBZ and diclobutrazol, DBZ) at the concentration of approximately 2.8 milligrams per 100 grams All standards was prepared in acetonitrile (99.8%) with distilled water (final ratio 3.5% : 96.5%, w/w). Into a 100 milliliters volumetric flask, approximately 2.8 milligrams (± 0.00001) of PBZ or DBZ standard was weighed and dissolved with 3.5 (± 0.00001) grams of acetonitrile Distilled water was then added to the flask but not reaching the mark. Then, the flasks were stored at 20 ˚C for about 20 minutes and readjusted to100 milliliters by distilled water with pasture pipette and thoroughly shake. A stock solution was used by spiking (paclobutrazol) and internal standard (diclobutrazol). The concentration of PBZ or DBZ solution should be maintained at 0.7 milligrams per kilogram sample after spiking into the samples. The stock standard was then used for working standard preparation with a range of concentrations at 0.5, 0.1, 0.05, 0.01, 0.005 milligrams per kilogram.

Preparation of spiking mango

Mangoes cultivars Tommy Atkins, Kent, Haden, and Palmer at green mature and eating-ripe stage were bought from the market in Stuttgart, Germany for the investigation. The 1,000 grams of mango was randomly selected, chopped and homogenized by Grindomix GM 200 (3 times, 10 seconds for 7500 U/minute). One to three grams of mango puree were taken to evaluate the water content in the sample by Infrared-moisture content machine (Sartorius MA 40) for 3 replicates. In addition, total soluble solid was measured by digital refractometer in 3 times. Approximately 250-300 grams (± 0.01) of mango pulp was weighed into 500 milliliters of glass bottle and stirred by magnetic stir bar. The samples were then spiked with paclobutrazol at the concentration of 0.7 milligrams per kilogram sample and mixed well for 3 minutes. The 100 grams of mango samples were transferred into plastic box, closed with a lid and frozen at - 80 ˚C until analysis. As shown in Figure 6.1. The data obtained was calculated using MS-excel® software (see appendix). Three replications were done for each treatments and tests.

The extraction procedure of paclobutrazol from the mango pulp

A puree of 100 grams was exactly weighed (± 0.001) into a 500 milliliters of glass bottle, the internal standard (diclobutrazol [DBZ] 0.7 milligrams per kilogram sample), and magnetic stir bar were added and homogenized for 5 minutes. Acetonitrile and water (Thier and Frehse, 1986) in ratio of 66.7 %:33.3 %, w/w; respectively, were added and the mixture was also homogenized for 10 minutes using magnetic stirring at high speed. Twenty grams of Celite 503 was added to supportable pulp mango during the filtration and stirred for 2 minutes. The extraction bottle was weighed and tared. 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, 66.7 %:33.3 %, w/w), rinsing of filter cake 2 times with 5 grams solution (ACN/H_2O, 66.7 %:33.3 %, 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 66.5 % weight loss. The addition of acetonitrile was used to adjust the ACN/H_2O ratio (3.5 %: 96.5 %). 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 6.2.

Optimization of paclobutrazol extracts time in extraction mango procedure

The sample (Tommy Atkins) was taken at approximately 250-300 grams (± 0.01) and then spiked with paclobutrazol at the concentration of 0.7 milligrams per kilogram sample and mixed well by magnetic stirring. After that 100 grams of each sample were taken 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 (66.7 %:33.3 %, w/w), homogenized by stirring for various times ,10, 20, 30, 45 and 60 minutes, then added the Celite 503 and filtrated. The mixture solution was evaporated under the vacuum (30 ˚C, <150 millibars) but not until dryness, only until ACN has been removed 66.5 % weight loss. The supernatant liquid (1 milliliter) was collected and diluted to 10 milliliters (ACN/H_20 3.5 %:96.5 %, w/w) as described above (Figure 6.2). 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.

6.3.3 Method validation

Recovery of paclobutrazol in mango pulp

The stock solution of paclobutrazol was prepared as described above. This stock solution was further diluted to obtain standard solutions of 0.05, 0.1, and 0.5 milligrams per kilogram. Recovery study of paclobutrazol was carried out in mango cultivar Tommy Atkins. The sample was weighed at 250-300 grams (± 0.01) and then spiked with paclobutrazol at the concentration of 0.5, 0.1, and 0.05 milligrams per kilogram sample and mixed well by magnetic stirring. After that 100 grams of each sample were taken 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 (66.7 %:33.3 %, w/w) and filtrated. The mixture solution was evaporated under the vacuum (30 ˚C, <150 millibars) but not until dryness, only until ACN has been removed 66.5 % weight loss. The supernatant liquid (1 milliliter) was collected and diluted to 10 milliliters (ACN/H_20 3.5 %:96.5 %, w/w) as described above (Figure 6.2). 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.

Limit of detection of paclobutrazol in mango pulp

The stock solution of paclobutrazol was prepared as mentioned above. This stock solution was further diluted to obtain standard solutions of 0.005, and 0.01 milligrams per kilogram. The Detection of limit study of paclobutrazol was carried out in mango cultivar Tommy Atkins. The sample was taken 250-300 grams (± 0.01) and then spiked with paclobutrazol at the concentration of 0.005, and 0.01 milligrams per kilogram sample and mixed well by magnetic stirring. After that 100 grams of each sample were taken 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 (66.7 %:33.3 %, w/w) and filtrated. The

mixture solution was evaporated under the vacuum (30 ˚C, <150 millibars) but not until dryness, only until ACN has been removed 66.5 % weight loss. The supernatant liquid (1 milliliter) was collected and diluted to 10 milliliters (ACN/H_20 3.5 %:96.5 %, w/w) as described above (Figure 6.2). 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 (EI) mode. The dilution of paclobutrazol in ratio 1: 10, 1:5 and 1: 4 were evaluated.

The effect of ripening stage and cultivars on recovery percentage

Mangoes cultivars Kent, Haden, Palmer at eating-ripe stage were bought from the market in Germany for the investigation of the impact of cultivars and reproducibility. For the investigation of the effect of ripening stage on the recovery of paclobutrazol, mature green mango cultivar Tommy Atkins was selected. They were ripened for 7 days at an ambient average temperature at 22.5 ˚C, average relative humidity at 30.7 %. One kilogram of fruit was daily withdrawn from the box and assessed for the quality. The pulp was analysed for total soluble solids (TSS), titratable acidity (TA), ratio of TSS/TA, pH and texture. Mango pulp of each variant was chopped and frozen at - 20 ˚C prior to the analysis of paclobutrazol.

To determine the percentage recovery of paclobutrazol in mango pulp, each sample was taken 250-300 grams (± 0.01) and then spiked with paclobutrazol at the concentration of 0.7 milligrams per kilogram sample and mixed equally by magnetic stirring (Figure 6.1). After that 100 grams of each sample were taken 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 (66.7 %:33.3 %, w/w) and filtrated. The mixture solution was evaporated under the vacuum (30 ˚C, <150 millibars) but not until dryness, only until ACN has been removed, 66.5 % 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 6.2). 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 (EI) mode.

Stability of SPME fiber

During the analysis of paclobutrazol, the number of an injection was counted and recorded in the time table. It was made to a profile of fiber used. Every day before starting the analysis, daily injection of the standard was needed with the concentration of paclobutrazol at 0.01 milligram per kilogram coupled with diclobutrazol at 0.1 milligram per kilogram (internal standard). Then the peak areas of each standard were plotted as a graph for controlling. When the peak areas of both standards were dropped lower than those of the previous detections, it can be predicted the fiber degenerate. However, this is mainly based on the increase in the peak areas of paclobutrazol. Then, the new fiber can be replaced. In this way, it was easily used to control and checked the stability of SPME fiber. A blank desorption was run before daily standard injection for checking the effect of carry over together.

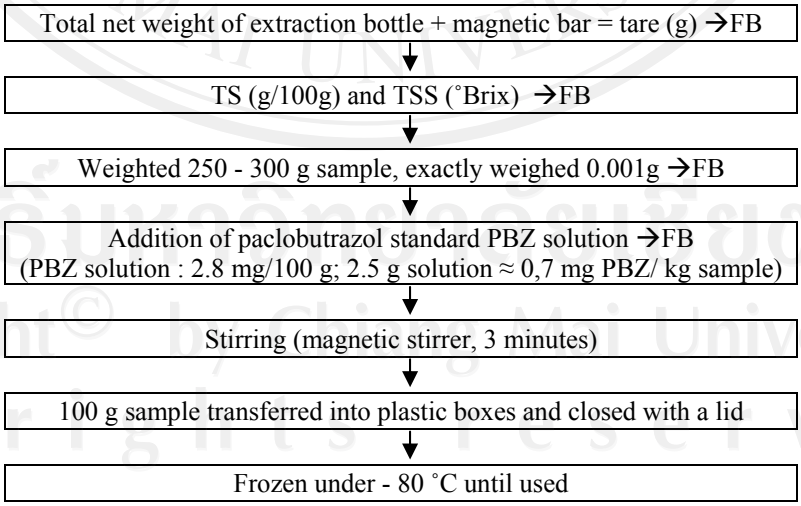

Figure 6.1 Scheme of spiking mango samples with paclobutrazol standard.

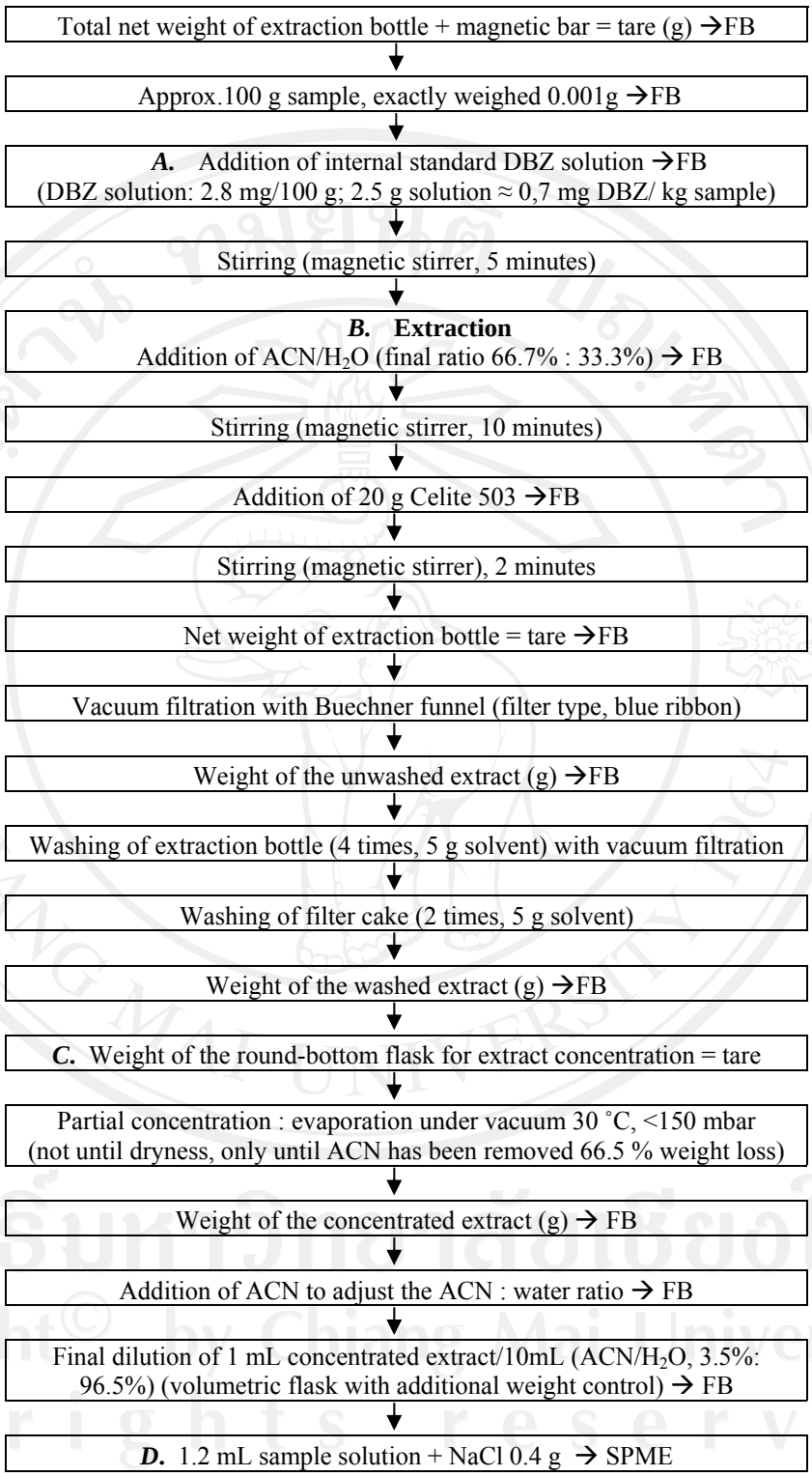

Figure 6.2 Scheme of mango sample extraction procedure. *A*. addition of internal standard, *B*. sample extraction, *C*. concentration step, *D*. SPME extraction.

6.4 Results

6.4.1 Preliminary extraction of PBZ in mango fruit

As for an experiment preliminary extraction of PBZ in mango fruit, a graph of the responding to paclobutrazol and diclobutrazol were found at the same retention time when compare with the spectra of specific peaks associated with standard data in Chapter 5. This chapter was to find out the information of paclobutrazol and internal standard. The spectrums were identical which indicated that the same compound was presented. In addition, the areas of peak were not significantly different. This was confirmed that the solvent ratio used was a good solution for paclobutrazol residue extraction in the pulp of mango.

6.4.2 Optimal GC-MS condition

The optimization of adsorption time, desorption time and cleaning up time of SPME fiber were observed at 60, 6, and 10 minutes, respectively and used as same as in the chapter 5.

Optimization of paclobutrazol extracts time in extraction mango procedure

The optimization of paclobutrazol extracts time in procedure illustrated that the recoveries were occurred at various times as above 100 % and above the theoretical concentration line of paclobutrazol (Figure 6.3). Therefore extract time was selected at 10 minutes.

6.4.3 Method validation

Recovery of paclobutrazol in mango pulp

For recovery experiments, three different concentrations were spiked at 0.5, 0.1, and 0.05 milligrams per kilogram sample. The analysis of paclobutrazol was

carried out in mango pulp as described. The recovery efficiency data were shown in Table 6.1 by analyzing uncontaminated mango extracts. The recoveries of the paclobutrazol were very good and higher than 85.0 % in most cases. However, the standard deviation (S.D.) and coefficient of variant (CV) at the lowest level of paclobutrazol (0.05 milligrams per kilogram sample) of the spiked sample was relatively high (18.4 % and 15.85 %, respectively), but it could be in an acceptable range. In addition, the expectant paclobutrazol in comparison to the mean detectable paclobutrazol were not significantly different. No substrate interferences were observed in any sample as compared to the control (data not shown). The quantification above 0.01 milligram per kilogram sample gave high percent recovery which ranged between 80.9 % and 116.1 % (Table 6.1, 6.2).

Limit of detection of paclobutrazol in mango pulp

The study of detection limit showed that two different concentrations were spiked at 0.01 and 0.005 milligrams per kilogram sample. It was found that the concentration of 0.005 milligrams per kilogram was detected. Therefore, the mean recovery of paclobutrazol was below 56 % in both dilution ratios. However at the same concentration, the dilution sample ratio 1:4 showed the highest recovery at 81.5 %. At the concentration of 0.01 milligram per kilogram was observed high mean recovery in most of the dilutions (>80 %) and the mean detectable of paclobutrazol were rather similar in different dilution. The data were shown in Table 6.2. Therefore, the limit of quantification was achieved at the concentration of 0.01 milligram per kilogram fresh mango mesocarp. The quantitative analysis of the linearity of response of the concentration is displayed in Figure 6.4. 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 mango pulp.

The effect of ripening stage and cultivars on recovery percentage

Paclobutrazol contents in mango pulp of the three cultivars studied and their recovery percentages are presented in Figure 6.5. The paclobutrazol recovery

percentage was in the range of 62.81 % – 90.67 % depending on cultivars. The minimum value was found in cultivar Palmer at 62.81 %. This was probably due to the higher TSS/TA ratio and lower texture value compared to other cultivars (Table 6.3). Higher reducing sugar, pectin or fiber contained in pulp, may cause substrate interferences by increased viscosity, which could be observed during the extraction. The extraction efficiency at various ripening stages of mango cultivar. Result of the Tommy Atkins is shown in Figure 6.5. It was found that the recovery percentage of paclobutrazol was almost the same during the first 3 days of ripening in the range of 96.17 % - 99.26 % and rapidly dropped in day 4 when mangoes attained the eating stage of ripeness at mean 85.29 %. Furthermore, the texture firmness also decreased approximately 50 % in the same time (Table 6.4). However, paclobutrazol was not found in the control treatment (blank, data not shown). A good repeatability from each cultivar repetitive determination of recovery has been achieved (CV ≤ 20 %) for all analytes. The reproducibility and repeatability of the method were in term of the standard deviation (S.D.) and coefficient of variant (CV) in all case (Figure 6.5).

Table 6.1 Recovery of paclobutrazol from fortified processed with Tommy Atkins (adapted from Reintjes, 2005)

Spiked (mg/kg)	Mean Recovery (%)	S.D. Recovery	CV (%)	PBZ (mg/kg mango)		
				Expected	Mean Detected	n
0.5	85.7	9.6	11.18	0.4917	0.422	3
0.1	94.8	4.6	4.85	0.1056	0.100	3
0.05	116.1	18.4	15.85	0.0521	0.061	4

Table 6.2 Limit of detection in analysis of Tommy Atkins samples (Reintjes, 2005).

Spiked	Dilute	PBZ (mg/kg mango)				Mean
		Expected	Mean	S.D.		Recovery
(mg/kg)	Ratio	Results	Detected	Detected	n	(%)
0.01	1:10	0.0111	0.0090	0.001	4	80.9
	1:5	0.0111	0.0096	0.002	4	86.5
	1:4	0.0111	0.0096	0.002	4	86.4
0.005	1:10	0.0056	0.0029	0.003	4	52.3
	1:5	0.0056	0.0031	0.005	4	55.9
	1:4	0.0056	0.0046	-	1	81.5

Table 6.3 Properties of ripen mango cultivars. (Kent, Haden and Palmer)

Cultivar	pH	TSS (˚Brix)	TA(pH 8.1)(g/L)	TSS/TA	Texture (N/100g)
Kent	3.60	15.67 ± 0.05	7.93 ± 0.002	19.77	3447.99 ± 224.94
Haden	3.80	15.77 ± 0.04	6.18 ± 0.014	25.51	1458.94 ± 192.06
Palmer	4.64	14.62 ± 0.06	1.44 ± 0.001	101.67	163.52 ± 12.72

Table 6.4 Properties of Tommy Atkins at various ripening stages

Tommy Atkins	pH	TSS (˚Brix)	TA(pH 8.1)(g/L)	TSS/TA	Texture (N/100g)
day 0	3.28	13.42 ± 0.04	11.99 ± 0.03	11.90	7285.800 ± 409.59
day 1	3.78	12.84 ± 0.02	6.14 ± 0.01	20.90	3988.116 ± 245.49
day 2	3.68	13.81 ± 0.01	6.42 ± 0.10	21.51	4579.641 ± 410.14
day 3	3.66	13.37 ± 0.06	7.05 ± 0.01	18.96	2986.132 ± 292.92
day 4	3.80	14.44 ± 0.06	4.22 ± 0.01	17.65	1483.683 ± 177.82
day 7	4.26	15.12 ± 0.08	3.06 ± 0.01	49.35	1121.162 ± 116.10

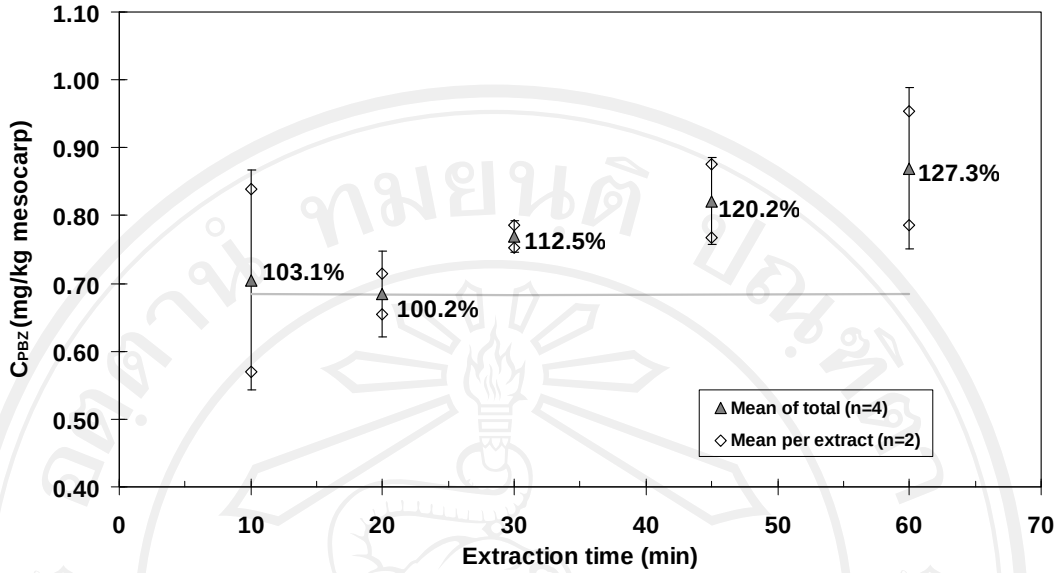

Figure 6.3 Recovery percentages for optimizing extract time in extraction mango procedure (adapted from Reintjes, 2005).

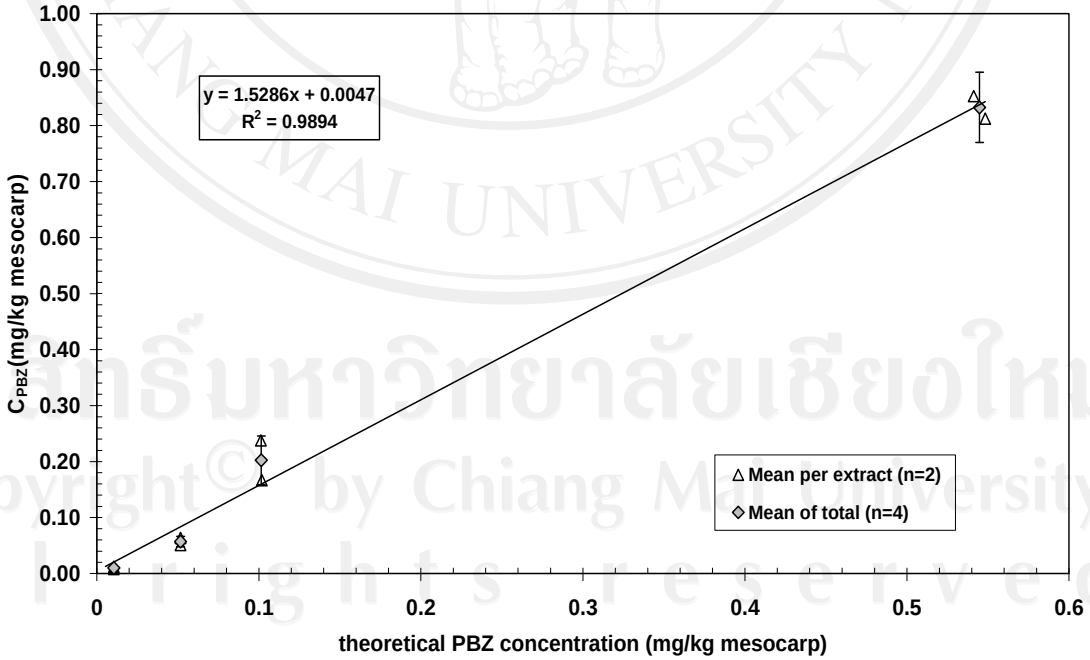

Figure 6.4 Linearity plot by means of the paclobutrazol standard addition method (adapted from Reintjes, 2005).

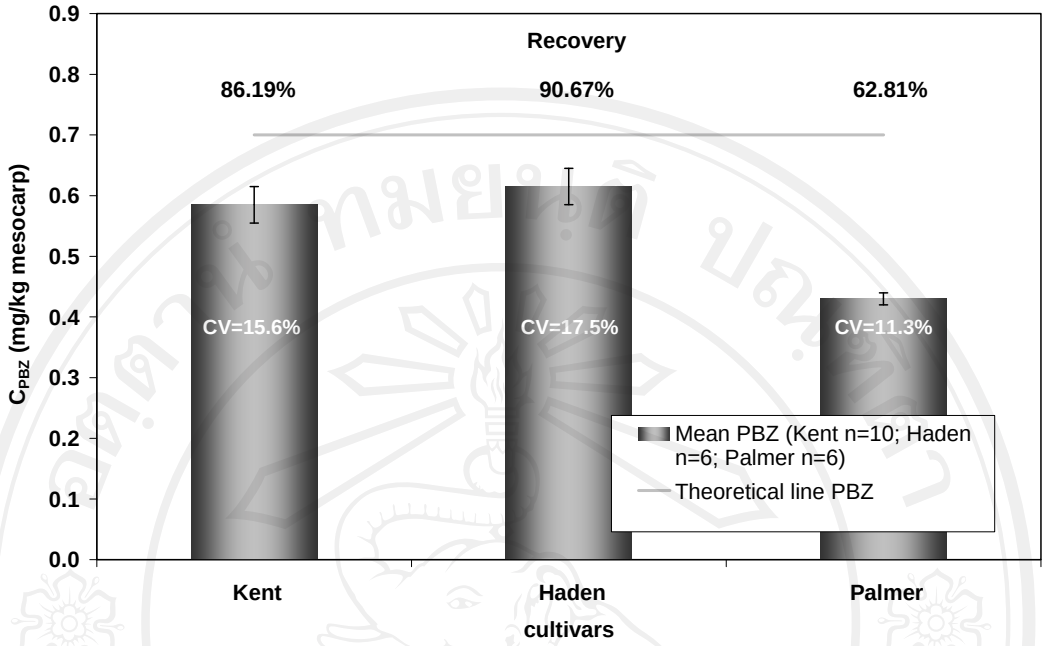

Figure 6.5 Recovery percentages of additive PBZ as affected by various mango cultivars Kent (n = 10), Haden (n = 6) and Palmer (n =6).

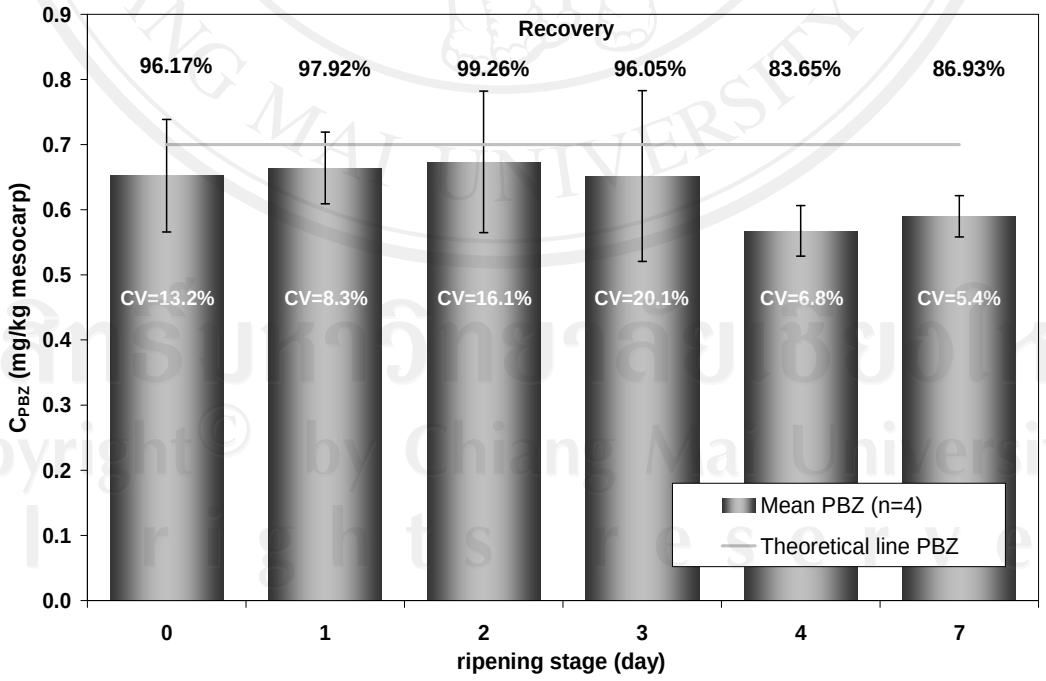

Figure 6.6 Recovery percentages of additive PBZ as affected by Tommy Atkins ripening stage (n = 4 per day).

Stability of SPME fiber life time

The data were recoded in everyday basis, especially the daily standard, and plotted a graph which was used to control and check the stability of SPME fiber. Before the optimization of SPME, it takes 1 hour to clean the fiber at 300 ˚C, a number of an injection in only one fiber was found about 20-40 times. After the change of SPME condition (optimization of SPME), particular in the clean up of fiber, it was found that one SPME fiber could be used for analysis more 80 injections. As shown in Figure 6.7. The fiber will be deteriorated when peak areas of paclobutrazol decreased below those of the previous data or also together with diclobutrazol data. However, the alteration of diclobutrazol was not constantly decent which in principal it should be used the peak areas of paclobutrazol to arbitrate the cessation of fiber. A blank desorption experiment was run, especially for the tests of paclobutrazol at a very low concentration. It often observed that no carryover from previous run was found.

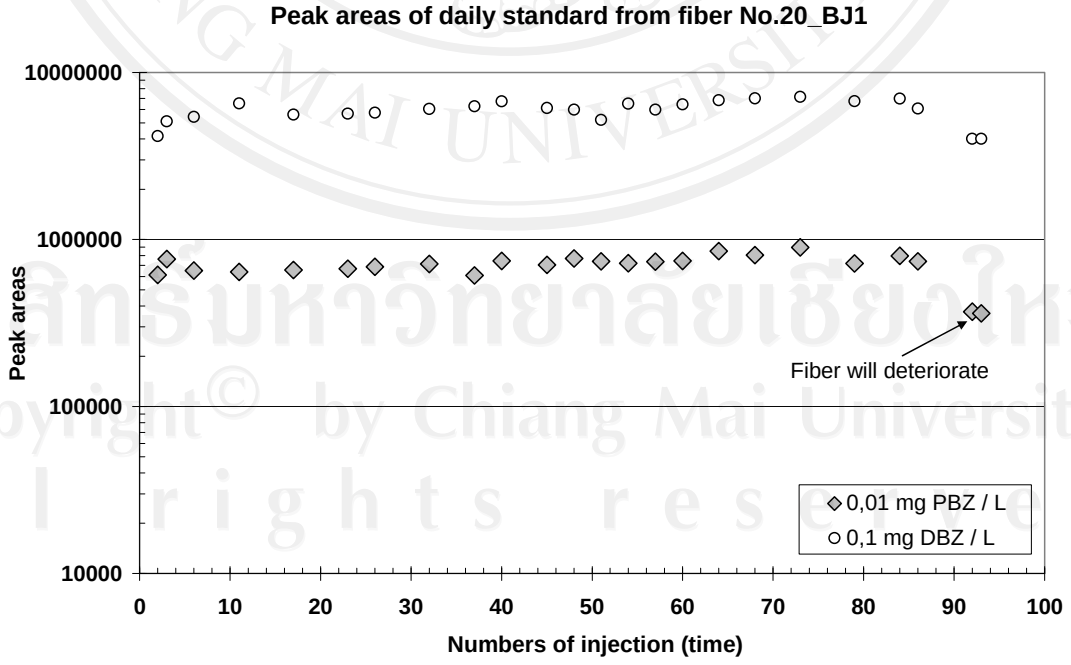

Figure 6.7 Stability of SPME fiber controlling by daily standard injection of peak areas.

6.5 Discussion

The preliminary test showed that paclobutrazol was extracted in mango pulp experiment. The aim of this test was only checking the efficiency of extract solution (acetonitrile with water 35:65, recommended by Crook, 1999) which was compared between the retention time, peak areas, and the spectra of specific peaks in both substrates with standard data in chapter 5. It can be confirmed an excellent extract solution for paclobutrazol residue in mango pulp.

The optimization of extract time was occurred that the recovery was higher than 100 % in all cases. Ten minutes was chosen because of a good recovery and short time consuming. Concurrently, Thier and Frehse (1986) mentioned that the optimum of time for plants extraction should be in range of 10 to 20 minutes. However, there was also highly recovery of paclobutrazol at 20 to 60 minutes. In experimental recovery of paclobutrazol, the recovery efficiency could be accepted in all of cases. The standard deviation (S.D.) and coefficient of variant (CV) which was accepted at 20 % were rather high due to less repetition of each concentration obtained. A part of the limitation of detection was verified the method for extraction of paclobutrazol in mangoes pulp. The concentration 0.005 milligrams per kilogram sample was also detected but gave the lowest percentage recovery. However, when the dilution ratio was changed from 1:5 to 1:4, but only one injection, it gave the highest recovery as 81.5 % (Table 6.2). As mentioned by Simplício and Boas (1999), the complexity of the fruit matrix makes it difficult to obtain a quantitative of pesticides. Nonetheless, the decrease in concentration of the interfering components by simple dilution of the sample makes possible the quantification of pesticides. Moreover, it should be emphasized that when quantitative results have to be obtained the use of calibration by external standard prepared with ultra purified water (even after sample matrix dilution) is not always feasible. Most authors recommended the use of internal/surrogate standard addition method for the accurate quantitation of samples (Beltran *et al*., 2000).

Also Bicchi *et al*. (2001) found that the method was described for the simultaneous determination of diclobutrazol, flusilazole, flutriafol, hexaconazole, paclobutrazol, and tetraconazole in apple and pear pulps used in baby food at a limit of 0.01 milligram per kilogram. Apple and pear pulp are subjected to selective solid-phase microdispersion (SPMD) with SPE-ED Matrix-38. The extracts are then analyzed with liquid chromatography and ultraviolet detection, using an octadecylsilane column with a gradient-programmed acetonitrile-water mobile phase. Recoveries were determined by spiking apple and pear pulps with the 6 pesticides under investigation at 0.1, 0.05, 0.03, and 0.01 milligrams per kilogram. Six determinations were performed at each level for each pesticide. Recoveries were ≥ 70 % at the 0.01 milligram per kilogram level. In addition, Sancho *et al*. (2003) reported that a rapid and sensitive liquid chromatography /electrospray ionization/tandem mass spectrometry (LC-ESI-MS-MS) method has been developed for the determination of the paclobutrazol in pear samples. Extraction was performed with methanol by using a high-speed blender Ultra-Turrax, and 10 microlitres of pear extract was directly injected in the LC- ESI-MS-MS system without any previous sample treatment. The highest sensitivity of the method was achieved under MS-MS conditions obtaining a limit of detection of 0.7 micrograms per kilogram and quantification limit of 5 microgram per kilogram, with a run time of only 5.5 minutes. Recoveries for paclobutrazol form spiked pear samples at 0.005, 0.05, and 0.5 milligrams per kilogram were around 82-102 % with relative standard deviations between 2 and 7 %. Arrebola *et al*. (2003) found that the method was validated in order to be applied to real samples for determination of 81 multiclass pesticides in fresh foodstuffs by a single injection analysis using gas chromatography-chemical ionization and electron ionization tandem mass spectrometry. Recovery in cucumber at two different fortification levels were evaluated and ranged between 73 % and 108 % for all pesticides. The relative standard deviation was lower than 22 % in all cases. The calculated limits of detection and quantification were lower than the maximum residue levels established by European legislations. Also Fussell *et al*. (2002) reported that the method validation data uncorrected for CPM and survival data for replicate days, such as paclobutrazol mean recovery 84.7 % and 74 % were observed.

The previously documentary has shown that the valid method analysis of paclobutrazol or other pesticides with different techniques and instruments, but also given recovery percentage as similar to this work. Although in different levels of concentrations in recovery and detection limit were obtained.

The impact of cultivars and ripening stages on recovery percentage were occurred. It may be due to the individual properties of cultivars, such as Palmer the high viscosity was observed during extraction practice. Higher reducing sugar, pectin or fiber contained in pulp, would cause substrate interferences by increased stickiness, also as same as in the influence of ripening stage. Thus to avoid these problems, the unripe flesh mango should be used for extraction and given highly recovery percentage. However, the ripen mango could also be used with the mean recovery was highly occurred (> 80 %).

The stability of SPME fiber under the optimization of SPME was increased those more than 80 injections for only one fiber. The SPME fiber capacity was enlarged in 2 times when compared with the condition used as stated in previously chapter “developing and optimizing GC-MS and SPME condition”, cleaning up process took 1 hour at 300 ˚C. Furthermore, the highest concentration of paclobutrazol in the spiked samples used for extraction was also destroyed and decreased the capacity of fiber. The simple dilution of sample can solve the problem. The knowledge of this is the best way for assisting the researcher to know the critical life time of fiber.

6.6 Conclusion

The method described is suitable for the detection and quantification of paclobutrazol in mango fruit matrices. It was validated in a range below the maximum residue limits (MRL) of FAO (0.05 mg/kg), New Zealand and Japan (0.01 mg/kg) for the paclobutrazol studies.