CHAPTER 3

MATERAILS AND METHODS

3.1 Chemicals

1) Ethyl acetate, grade for organic residue analysis, J.T. Baker, Phillipsburg, USA

2) Isooctane, grade for organic residue analysis, J.T. Baker, Phillipsburg, USA

3) Hydrochloric acid (HCl), Pro analysis, Merck, Darmstadt, Germany

4) Methanol, grade for HPLC, J.T. Baker, Phillipsburg, USA

5) Anhydrous sodium acetate ($C_2H_3NaO_2$), Sigma-Aldrich, Saint Louis, USA

6) Ethyldiaminetetraacetic acid disodium salt (EDTA, purity 99%), Sigma-Aldrich, Poole, UK

7) Carbendazim standard ($C_9H_9N_3O_2$, 99.0%) , Dr. Ehrenstorfer GMbH, Augsberg, Germany

8) Mancozeb standard [$(C_4H_6MnN_2S_4)_xZn_y$, 72.0%] , Dr. Ehrenstorfer GMbH, Augsberg, Germany

9) SAX/PSA SPE (6 mL), International Sorbent Technology (IST) Ltd., Hengoed, UK

10) Filter paper No.1, Whatman, Piscataway, USA

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11) Polytetrafluoroethylene (PTFE) syringe filter (13 mm x 0.45 µm), Vertical Chromatography Co., Ltd, Bangkok, Thailand

12) High purity helium gas (99.999%), TIG, Bangkok, Thailand

13) Ultra high purity of nitrogen gas (99.999%), TIG, Bangkok, Thailand

14) High purity hydrogen gas (99.999%), TIG, Bangkok, Thailand

15) Air gas, TIG, Bangkok, Thailand

3.2 Apparatus

1) Chopper (DPA141), Moulinex, Ecully, France

2) Eluting vacuum (VacElut SPS-24), Varian, Palo Alto, USA

3) Vortex Mixer (VTX-3000L), Worldwide Trade Thai Co., Ltd, Bangkok, Thailand

4) High performance liquid chromatography (Model 1100 with UV detector), Hewlett-Packard/Agilent, Santa Clara, USA

5) C_{18} column (250 mm x 4.6 mm id, particle size 5 µm), Supelco Inc., Bellefonte, USA

6) Gas chromatograph (Model 6890 with flame photometric detector, FPD), Hewlett-Packard/Agilent, Santa Clara, USA

7) 5% phenylmethylpolysiloxane capillary column (30 m × 0.25 mm, 0.25 µm film thickness), Hewlett-Packard/Agilent, Santa Clara, USA

8) Micro pipette, Gilson Inc., Villiers le Bel, France

9) Evaporator (R-210), Buchi, Essen, Germany

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10) Ultrasonic cleaner (Cavitator), Mettler Electronics Corp., Anaheim, USA

3.3 Methods

3.3.1 Study site

The present study was a cross-sectional study designed to assess the exposure to carbendazim and mancozeb among consumers in Chiang Mai, Thailand. Consumers were considered as occupationally exposed subjects. Chiang Mai province is the largest province in northern Thailand and composed of 25 districts. Chiang Mai city is one of the intensive consumers in Chiang Mai province. Chiang Mai city was chosen to be the studied area by using conditional random sampling and the studied area is shown in Figure 3.1 and Chiang Mai city latitude and longitude are 18° 47' N and 98° 58' E.

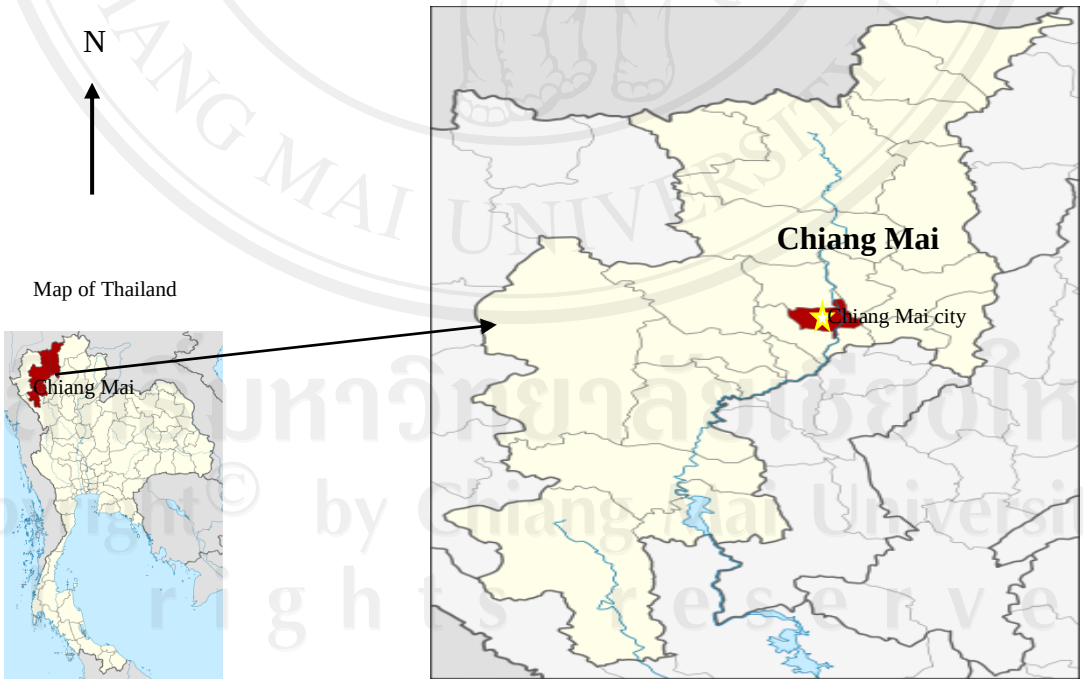

Figure 3.1 Map of the studied site

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3.3.2 Sample selection and collection

One kilogram of fresh vegetable samples was randomly selected from five fresh markets (Figure 3.2) in Chiang Mai city from August-October 2011. The vegetable samples including cauliflower, ginger, kale, cucumber, yard long bean, guinea-pepper, chili, pepper, and tomato were purchased and transported to Toxicology laboratory, Research Institute for Health Sciences, Chiang Mai University. All vegetable samples were chopped and blended into small pieces according to the Codex Alimentarius (2000). Their common and scientific names of vegetable samples were shown in Table 3.1

Table 3.1 Common and scientific names of vegetable samples from five fresh markets in Chiang Mai city

Common name	Scientific name
1. Cauliflower	*Brassica oleracea* L.
2. Chili	*Capsicum annuum* L.
3. Cucumber	*Cucumis sativa* L.
4. Ginger	*Zingiber officinale* Roscoe
5. Guinea-pepper	*Capsicum frutescens* L.
6. Kale	*Brassica albograba* Bailey
7. Pepper	*Piper nigrum* L.
8. Tomato	*Lycopersicon esculentum* Mill.
9. Yard long bean	*Vigna sesquipedalis* Koern.

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Figure 3.2 Five local fresh markets in Chiang Mai city

: Local fresh market

Source:

http://www.home.co.th/images/img_v/img_Directory/2012619132417_1pic.jpg

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3.3.3 Sample preparation

All vegetable samples were chopped and blended into small pieces according to the Codex Alimentarius (Codex Alimentarius, 2000). Ten g aliquots of well blended samples were kept frozen (-20 ºC) until analysis. The frozen vegetable samples were thawed and left to room temperature (25 ºC) before analysis.

3.3.4 Consumer participant enrollment

3.3.4.1 Sample size calculation

A sample survey is a process for collecting data on a sample of observations which are selected from the population of interest using a probability-based sample design. In sample surveys, certain methods are often used to improve the precision and control the costs of survey data collection. The sample size of consumption in the area of Suthep sub-district using probabilistic estimation equation defined as following. When the population size is known equation 3.1.

$$n = \frac{N Z_{\frac{\alpha}{2}}^{2} \sigma^{2}}{(N-1)e^{2} + Z\sigma^{2}} \qquad [3.1]$$

Where

n = number of sample

N = population

σ = standard deviation from other research

e = error value

$Z_{\alpha,2}$ = the critical standard score (Z) is the value for which the cumulative probability is 1 - alpha/2 = 1 - 0.05/2 = 1.96

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Suthep sub-district covers both urban and rural areas of 15 villages and one fresh market in the area of Suthep sub-district was studied. Fifteen villages of Suthep sub-district, including Cheng Doi, Namtok Huai Kaew, Kong Bin, Huai Sai, Ram Poeng, Pong Noi, Ton Kook, Ling Ha, Doi Sutep, Au Mong, Doi Pui, Phu Ping, Sanlom Joy, Mai Lang Mo, and Sai Kham villages. The total Sutep sub-district population was recorded at 16,885 people in 2012 (Tambon Suthep Municipality, 2012) and population numbers in Suthep sub-district described by village is shown in Table 3.2.

Table 3.2 Numbers of population in the Suthep sub-district of Chiang Mai, Thailand

Villages	**Population** (N)
1. Cheng Doi	713
2. Namtok Huai Kaew	449
3. Kong Bin	2,329
4. Huai Sai	973
5. Ram Poeng	1,920
6. Pong Noi	860
7. Ton Kook	1,351
8. Ling Ha	723
9. Doi Sutep	661
10. Au Mong	1,582
11. Doi Pui	1,386
12. Phu Ping	319

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Table 3.2 Numbers of population in the Suthep sub-district of Chiang Mai, Thailand (Cont'd)

Villages	**Population** (N)
13. Sanlom Joy	1,640
14. Mai Lang Mo	1,279
15. Sai Kham	700
Total	**16,885**

Source: Tambon Suthep Municipality, 2012.

3.3.4.2 Questionnaire preparation and testing

A structured questionnaire containing both open-ended and close-ended questions was developed for this study by the Ministry of Agriculture and Cooperatives (2006). The questionnaire was piloted with 30 consumers, who did not participate in the final study, and modified as necessary. The questionnaires covered questions about socio-demographic, socio-economic, health, including sex, age, body mass index (BMI), educational level, income, family members, occupation, congenital disease, self-cooking, the most popular vegetable and consumption and is shown in Appendix. This was a cross-sectional study that involved consumers selected randomly (≥35 years of age).

3.3.4.3 Data collection

Data collection was performed face-to-face interviewing consumers in the village in both urban and rural areas of 15 villages in Suthep sub-district, Chiang Mai city. The subjects included 244 consumers (144 females, 100 males) and numbers of consumers participated in the study from villages are shown in Table 3.3.

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Table 3.3 Numbers of consumers in the Sutep sub-district of Chiang Mai, Thailand

Villages	**Consumers**		
	Male	Female	Total
1. Cherg Doi,	3	4	7
2. Namtok Huai Kaew	7	9	16
3. Kong Bin	4	2	6
4. Huai Sai	4	4	8
5. Ram Poeng	5	9	14
6. Pong Noi	5	9	14
7. Ton Kook	11	9	20
8. Ling Ha	12	9	21
9. Doi Sutep	0	6	6
10. Au Mong	7	18	25
11. Doi Pui	18	26	44
12. Phu Ping	4	8	12
13. Sanlom Joy	6	11	17
14. Mai Lang Mo	7	11	18
15. Sai Kham	7	9	16
Total	**100**	**144**	**244**

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3.3.4.4 Data analysis

The optimization of extraction was used statistical analysis. Correlation analysis was carried out using the computer-based statistical program. Data were subjected to analysis of variance and means ± SD compared by Duncan Multiple Range Test (DMRT), which differences at $p \leq 0.05$ considered to be significant. Different of superscript letters a b and c in the top of results indicates significant.

3.3.5 Laboratory results

3.3.5.1 Methods for determination of carbendazim residue

3.3.5.1.1 Method for reproducibility, limits of detection and limits of quantitation

Pooled matrices collecting from several kinds of vegetables were spiked to obtain 5 levels including 0.05, 0.075, 0.10, 0.20 and 0.30 mg/kg, respectively. Spiked pooled samples were used to determine the critical parameters such as the linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision for method validation.

3.3.5.1.2 Method validation

Linearity was evaluated using 5 spiked pooled samples including 0.05, 0.075, 0.10, 0.20 and 0.30 mg/kg, respectively. The precision was evaluated by relative standard deviation (RSD) of five replications at each five concentrations. The accuracy was studied using 0.05 mg/kg spiked sample performing on the same day for five replications. Accuracy was then obtained by comparison of measured concentration to the spiked concentration and expressed as a percentage relative standard error value. LOD was obtained from the equation of RSD versus

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concentration. Finally, LOQ was determined as the lowest carbendazim concentration injected that based on a signal-to-noise ratio 10:1.

3.3.5.1.3 Sample extraction and analysis

The most commonly used analytical method for the analysis of carbendazim is liquid chromatography with UV detections and different extraction solvents. Several methods can be found in the literature for the analysis of carbendazim in the samples (fruits, vegetables, water, soils and wine). This method for the extraction and determination of carbendazim residues adapted from Pan et al. (2008) and QuEChERs (2008) methods. Thirty two vegetable samples including cauliflower, ginger, kale, cucumber, yard long bean, guinea-pepper, chili and tomato were purchased from five fresh markets in Chiang Mai city.

Weighed of 5.0 g aliquot sample and transferred to the extraction tube followed by the addition of 1 mL of 0.05 M HCl, 0.5 g of sodium acetate and 15 mL of ethyl acetate. Sample was then shaken for 1 min by using a vortex mixer and sonicated in the ultrasonic water bath around 25-28 ºC for 15 min. The extract was filtered through Whatman No.1 paper using a Buchner funnel. After that 7.5 mL of aliquot was filtered through a SAX/PSA dual-layer cartridge and washed with 10 mL of ethyl acetate and the filtrate was collected. The solvent was evaporated using a rotary evaporator and the dry residue was re-dissolved in 1 mL of HPLC mobile phase (methanol:water, 25:75, v/v) and then the residue was filtered through a 13 mm x 0.45 µm polytetrafluoroethylene membrane. Twenty µL of clean extract was injected into the HPLC-UV for carbendazim analysis. Flow diagram of sample preparation is shown in Figure 3.3

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Analytical conditions, the HPLC (Hewlett Packard Model 1100 binary pump and Model 1100 UV detector) was equipped with a variable UV detector set to 280 nm. Analytical column used was a C_{18} (250 mm x 4.6 mm id, particle size 5 µm) and the separation was carried out using a methanol-water (25:75). The flow rate was 1.0 mL/min and the injection volume was 20 µL.

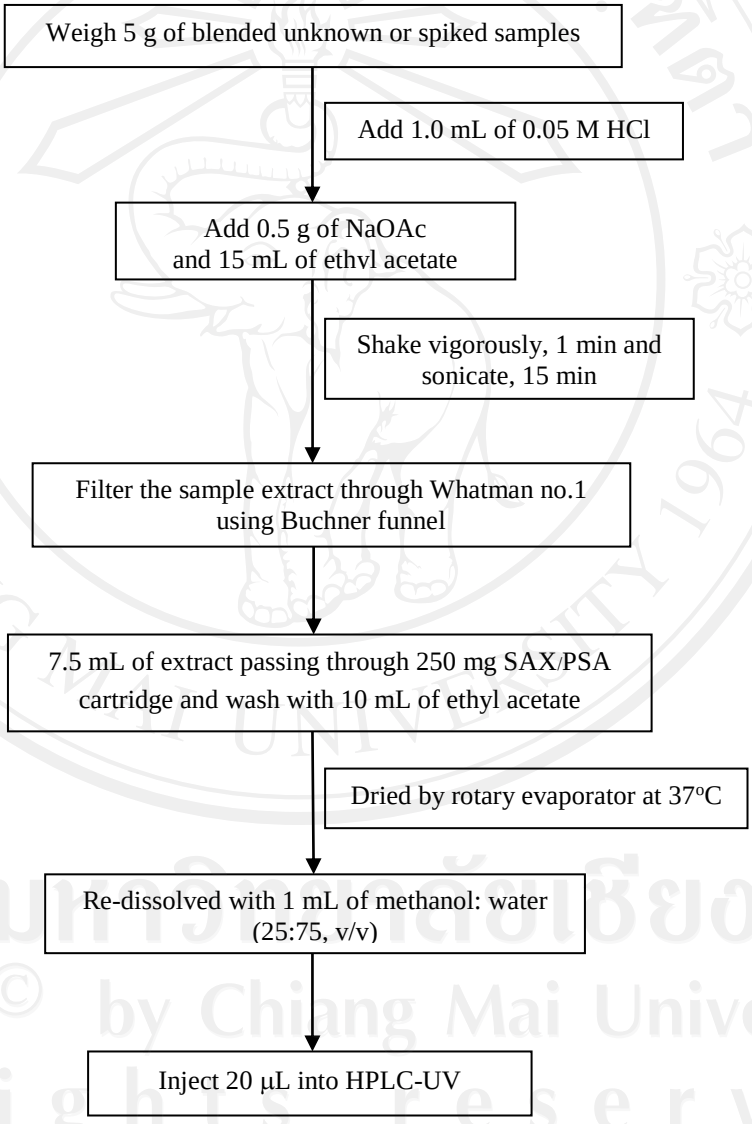

Figure 3.3 Flow diagram of sample preparation for carbendazim residue analysis

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3.3.5.2 Methods for determination of dithiocarbamate residue

3.3.5.2.1 Optimization of extraction

The parameters taken into consideration for optimization of extraction process were sample weight, concentration and volume of stannous chloride, volume of isooctane, and time of hydrolysis (sonication time). Mancozeb was employed as representative of the DTCs. In the following experiments, 1.0 mg/kg of mancozeb were spiked in the vegetable matrices including ginger, cucumber and pepper. The vegetable matrices was prepared by mixing ginger, cucumber and pepper at the ratio of 1:1:1 and then blended in a blender.

In the first experiments, the optimum sample weight of 5 and 10 g was compared. The vegetable matrices was spiked and sonicated in ultrasonic bath at 80°C for 40 min together with 1 mL of isooctane.

The second experiments were conducted to determine the optimum concentration and volume of the stannous chloride. To determine the optimum concentration of the stannous chloride, 5 g spiked vegetable matrices was studied at 0.1, 0.5, and 1.0% w/v in HCl 5 M. The optimum volume of the stannous chloride was determined by using 5 g of spiked vegetable matrice while 4, 5, and 6 mL of stannous chloride were added.

In the third experiments, the optimum volume of isooctane and sonication time. The volume of isooctane was added with 0.75, 1.0, and 1.5 mL and the optimum of ultrasonic extraction was sonicated for 10, 20, 30, 40, 50, and 60 min.

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3.3.5.2.2 Preparation of standard calibration curves

Mancozeb was spiked at 0.25, 0.50, 1.0, 1.5, 2.0 and 2.5 mg/kg in 5 g of vegetable matrices and then 4.0 mL of freshly prepared 0.5% $SnCl_2$ made in 5 M hydrochloric acid was added in each concentration followed by 1.0 mL of isooctane after the addition of the hydrolysis solution. Samples were heated in an ultrasonic water bath at 80°C for 40 min with continuous stirring and then allowed to room temperature (25°C). During cooling, the tubes were periodically hand-shaken to assist CS_2 into the isooctane layer and 1 µL of the isooctane layer was injected onto GC-FPD.

The GC-FPD system was operated in the sulfur and splitless mode. Separation was performed on HP-5 column (30 m x 0.25 mm i.d., 0.25 µm film thickness. The injector and detector temperature were set at 80 and 220°C, respectively. The column oven was programmed from 35°C (hold for 4 min at 35°C) to 120°C at 20°C/min (hold for 4 min at 120°C) and at 25°C/min to 220°C (hold for 2.5 min at 220°C). The carrier gas was helium (purity 99.999%) and set at a flow rate 1.1 mL/min.

3.3.5.2.3 Sample extraction and analysis

Total of fifty four samples including ginger, cucumber and pepper were purchased from five fresh markets in Chiang Mai city.

Weighed 5.0 g aliquot sample and transferred to the extraction tube followed by the addition of 4.0 mL of freshly prepared 0.5% $SnCl_2$ made in 5 M hydrochloric acid was added in each concentration followed by 1.0 mL of isooctane after the addition of the hydrolysis solution. Samples were heated in an ultrasonic water bath at 80 °C for 40 min with continuous stirring and then allowed to room

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temperature (25 °C). During cooling, the tubes were periodically hand-shaken to assist CS_2 into the isooctane layer and 1 µL of the isooctane layer was injected onto GC-FPD. Flow diagram of sample preparation is shown in Figure 3.4.

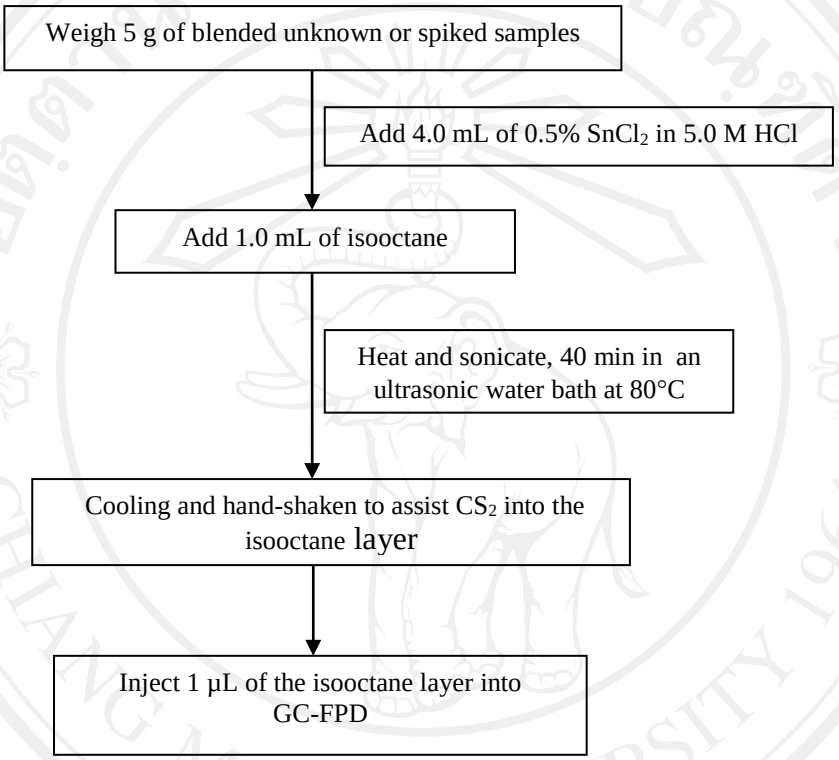

Figure 3.4 Flow diagram of sample preparation for mancozeb residue analysis

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3.3.6 Questionnaire data

The consumption data were obtained from 244 participants who were agricultural workers, general employee, traders, office workers, government officers and business owners, aged 35-65 years old by interviewing and using quantitative and frequency of vegetables consumption questionnaires. Eight levels of consumption frequency of 5 kinds of vegetables were interviewed including never intake, rarely intake (1-3 times a month), frequently intake comprised 1-2 times a week, 3-4 times a week, and 5-6 times a week, and usually intake included once a day, twice a day, and 3 times a day. Pictures of 5 proposed vegetables including tomato, cucumber, kale, cauliflower, and ginger showed their potential consumption sizes were used with the questionnaires by interviewer to identity the quantity of consumed vegetables.

3.3.7 Health risk assessment

3.3.7.1 Exposure assessment

The studied data of the quantity of carbendazim and mancozeb in the vegetables and the collected data of consumptions of the vegetables from interviewing were calculated to the exposure of carbendazim and mancozeb by using probabilistic estimation equation 3.2.

$$\Sigma \text{ Exposure or CDI} = \Sigma \text{ [Food consumption x Concentration]} \qquad [3.2]$$

Where exposure is the intake of carbendazim and mancozeb (mg/kg bw/day) or so called calculated daily intake (CDI), Food consumption is the weight of

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consumed vegetable per body weight of person per day, and Concentration (mean) the quantity of carbendazim and mancozeb in the vegetable (mg/kg).

3.3.8.2 Consumption data

Consumption data were collected by food frequency questionnaire. Consumption frequency of each vegetable was calculated to food intake in grams per day by the following for each frequency option: never intake = 0, rarely intake (1-3 times a month) = 0.099, frequently intake comprised 1-2 times a week = 0.26, 3-4 times a week = 0.53, and 5-6 times a week = 0.756, and usually intake included once a day = 1, twice a day = 2, and 3 times a day = 3. Daily grams of consumption of each vegetable were calculated by summing in each vegetable per day and obtaining the mean of all weighing days. Food consumption is the weight of consumed vegetable per body weight of person per day.

3.3.8.3 Risk assessment

The CDI of each vegetable and the consumer's acceptable daily intake (ADI) were calculated as the risk assessment of carbendazim and mancozeb using the following equation 3.3.

$$\textbf{Risk Assessment (\%ADI)} = \frac{\textbf{CDI x 100}}{\textbf{ADI}} \qquad \textbf{[3.3]}$$

Where risk assessment is the risk of carbendazim and mancozeb of the vegetable, CDI is the exposure of carbendazim and mancozeb residue from consumed vegetable (g/day), and ADI is consumer's acceptable daily intake of carbendazim and

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mancozeb (mg/kg bw/day). ADI of carbendazim and mancozeb that set at 0.03 mg/kg bw/day (FAO/WHO, 2005)

The Joint FAO/WHO Expert Committee of Food Additives (JECFA) establish the acceptable daily intake (ADI) which is a measure of the amount of a specific substance in food that can be ingested on a daily basis over a lifetime without an appreciable health risk. If the exposure of carbendazim and mancozeb are higher than 100% of ADI, it means that it has risk to human health (not safe to consume). On the other hand, if it lower than 100% of ADI it has no risk to consumer health (safe to consume).

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