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Contributed Paper

A Sensitive Method for Determination of Carbendazim Residue in Vegetable Samples Using HPLC-UV and Its Application in Health Risk Assessment

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ABSTRACT

Carbendazim is a fungicide widely used in vegetable growing and its residue was often detected. We developed a sensitive method using high performance liquid chromatography-UV detection (HPLC-UV) for determining carbendazim residue in vegetables and assessed the health risk from contaminated vegetable consumption. The consumption data was collected from 244 participants aged 35-65 years living in Suthep subdistrict of Chaing Mai city while vegetable samples were collected from Chiang Mai city fresh markets during August to October 2011. The developed method provided good recoveries of carbendazim from spiked pooled vegetable samples ranged from 92.5 % to 96.0 % with the relative standard deviation (RSD) of 2.1 % to 5.9 % at spiked levels of 0.05-0.30 mg kg^{-1}. The limit of detection (LOD: 0.003 mg kg^{-1}) as well as the limit of quantitation (LOQ: 0.030 mg kg^{-1}) of the developed method is sensitive to detect carbendazim residue in vegetables far below the Codex Maximum Residue Limits (MRLs) such as 0.05 mg kg^{-1} set for cucumber. The health risk assessment of carbendazim from consumption of 5 common kinds of vegetables, i.e. tomato, cucumber, kale, cauliflower, and ginger, was performed by comparing calculated daily intake (CDI) with acceptable daily intake (ADI). It was found that the CDI of carbendazim residue from consumption of tomato (64.1 %), cucumber (59.6 %), and kale (56.7 %) were greater than 50 % of ADI while of cauliflower (19.3 %), and ginger (0.9 %) were much lower. The present results showed that consumption of some contaminated vegetables may pose the health risk though the commination may vary from season to season. Furthermore, the developed method could be used to survey carbendazim

residue in vegetables as well as fruits by using HPLC-UV which is a common apparatus available in toxicology laboratory.

Keywords: carbendazim residue, vegetables, consumption, HPLC-UV, health risk assessment

1. INTRODUCTION

In recent years, the total amount of pesticides used has increased worldwide. In Thailand, the Department of Agriculture (DOA) reported that pesticides were increased 1.2 fold in 5 year time from 110,000 tons in 2008 to 134,000 tons in 2013. The major abundant was herbicides, insecticides and fungicides, respectively [1]. Some pesticide residues were reportedly detected in vegetables, fruits, and soil [2-3]. Benzimidazole fungicides which the main compound is carbendazim are systemic pesticides widely used in agriculture for pre- and post-harvest protection of crops against fungal diseases [4] and structure of carbendazim is shown in Figure 1. Carbendazim is toxic to humans, animals and plants. The toxicity produces rapid effects on meiotic spermatocytes and latent effects on spermatids, leading to morphological abnormalities and failure of spermatogenesis [5]. It was also found that subchronic administration of carbendazim induced testicular alterations, spermatogenic inactivity and embryotoxicity [6]. Over the years, research in the field of carbendazim residue analysis as well as other pesticides in food has continuously expanded according to food safety policy and techniques available for determining their content in different fruits and vegetables [7-9]. Therefore, it is still of interest to develop a sensitive and low cost analytical method that suitable and applicable for toxicology laboratory especially in developing country.

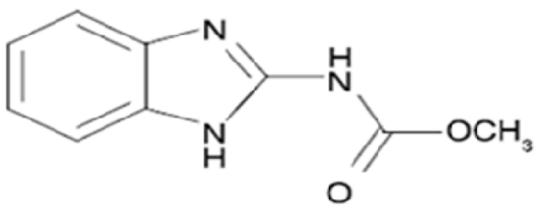

Figure 1. Structure of carbendazim.

The common used analytical techniques for the analysis of carbendazim include liquid chromatography with UV [4, 10], diode-array [11], fluorescence [12, 13] or mass spectrometric detections [14]. Various extraction solvents such as acetone [15], acetonitrile [16], methanol [4], ethyl acetate [14, 17], and dichloromethane [18] have been used to extract carbendazim residue followed by homogenizing, and shaking by using sonication [17]. Moreover, a solid-phase extraction (SPE), using C_{18} bonded silica procedure, has been employed for isolation [19]. Pan *et al*. (2008) reported that mobile phase modifiers such as methanol-water or acetonitrile-water were required to improve the peak shape and/or resolution [20]. There was effective absorption of interferences on strong anion exchange/ primary secondary amine (SAX/PSA) dual-layer SPE for the HPLC condition.

A health risk assessment, i.e. carbendazim,

is the process to estimate the nature and probability of adverse health effects in humans who may be exposed to carbendazim in contaminated food, now or in the future. Basically, there are four major steps in human health risk assessment including hazard identification, dose-response assessment, exposure assessment, and risk characterization. The first and the second steps are processed following the Codex Alimentarius Commission (Codex) which evaluates the hazards by measuring both quality and quantity. The Joint Meeting of Food and Agriculture Organization and the World Health Organization (FAO/WHO) on Pesticide Residues (JMPR), is responsible for reviewing residue and analytical aspects of the pesticides in foods. The health risk assessment of carbendazim exposure will be compared with the acceptable daily intake (ADI) which is a measure of the amount of a certain substance in food that can be ingested on a daily basis over a lifetime by human without appreciable health risk. The ADI of carbendazim is 0.03 mg kg^{-1} bw day^{-1} [21]. Therefore, the result of human health risk assessment from contaminated carbendazim residue in vegetables could be useful for food safety policy.

2. MATERIALS AND METHODS

2.1 Chemicals

Carbendazim (99% purity) was from Dr. Ehrenstorfer (Augsburg, Germany). Stock solution was prepared by dissolving 1 mg of carbendazim in 25% methanol up to 100 mL (10 μg mL^{-1}). Working solutions for spiking in validation studies were prepared by diluting 1.2, 1.9, 2.5, 5.0 and 7.5 mL of stock solution in 25% methanol up to 10 mL. The obtained concentrations of working solutions were 1.2, 1.9, 2.5, 5.0 and 7.5 μg mL^{-1}, respectively.

SAX/PSA dual-layer SPE bulk materials and 500 mg/6 mL SPE cartridges were from Varian Inc. (Harbor, USA.). Organic solvents such as ethyl acetate and methanol (HPLC grade) were from J.T. Baker (NJ, USA). Sodium acetate was from Sigma-Aldrich (MO, USA.) and hydrochloric acid (analytical grade) was from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water purification system, ELGA UHQ PSII (Bucks, UK). Mobile phase was filtered through a 13 mm × 0.45 μm polytetrafluoroethylene VertiClean from Vertical Chromatography (Nonthaburi, Thailand).

2.2 Instrumentation

The HPLC (Agilent 1100, USA) was equipped with a variable UV detector set to 280 nm. Analytical column was C_{18} (250 mm × 4.6 mm i.d. and 5 μm particle size (Supelco Inc., Bellefonte, PA, USA.). The mobile phase consisted of methanol-water (25:75, v/v). The flow rate was 1.0 mL min^{-1} and the injection volume was 20 μL.

2.3 Sample Preparation

One kilogram of fresh vegetable samples was randomly selected from four fresh markets in Chiang Mai city from August to October 2011. Thirty two vegetable samples including cauliflower, ginger, kale, cucumber, yard long bean, guinea-pepper, chili 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 [21]. Ten grams' aliquots of well blended samples were kept frozen (-20 °C) until analysis. The frozen vegetable aliquot samples were thawed and left to room temperature (25 °C) before analysis. 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^{-1}, 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.

2.4 Method Validation

Linearity was evaluated using 5 spiked pooled samples including 0.05, 0.075, 0.10, 0.20 and 0.30 mg kg^{-1}, 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. Limit of detection (LOD) was obtained from the equation of RSD versus concentration. Limit of quantitation (LOQ) was defined as 10 times of the LOD.

2.5 Sample Extraction

Sample extraction involves two steps: extraction and clean-up with SPE.

Weighed 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 using a vortex mixer and sonicated in the ultrasonic water bath at 25-28 °C 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 5 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 × 0.45 μm polytetrafluoroethylene membrane. Twenty microliters of clean extract was injected into the HPLC-UV for carbendazim analysis.

2.6 Data Collection for Human Health Risk Assessment

Suthep subdistrict of Chiang Mai city was chosen to be the studied area by using conditional random sampling. Both urban and rural areas of 15 villages, and one market in the area of Suthep subdistrict were studied. The consumption data was obtained from 244 participants who were farmers, general employees, traders, office workers, government officers and business owners, aged 35-65 years old. Data was collected using a questionnaire-based personal interview. Pictures of 5 kinds of vegetables including tomato, cucumber, kale, cauliflower, and ginger and their potential consumption were shown while interviewing. The quantity and frequency of vegetables consumption were estimated from eight answers, including 'never intake', 'rarely intake (1-3 times a month)', 'frequently intake (1-2 times a week)', '3-4 times a week', and '5-6 times a week', and 'once a day-most days', 'twice a day', and '3 times a day'.

2.7 Statistical Analysis

Data of concentration of carbendazim residue and consumption quantity of vegetables from interviewing were calculated for the exposure of carbendazim by using Probabilistic Estimation equation (1) as following.

$$\Sigma \text{ Exposure} = \Sigma \text{ (Food consumption} \times \text{Concentration)} \quad (1)$$

where exposure is the intake of carbendazim (mg kg^{-1} bw day^{-1}) or so called calculated daily intake (CDI), Food consumption is the

weight of consumed vegetable per body weight of person per day (mg kg $bw^{-1}day^{-1}$), and Concentration (mean) of carbendazim in the vegetable (mg kg^{-1}).

The CDI of each vegetable, and the acceptable daily intake (ADI) were calculated as the risk assessment of carbendazim using the equation (2) as following.

$$\text{Risk Assesment (\% ADI)} = \frac{\text{CDI} \times 100}{\text{ADI}} \quad (2)$$

where risk assessment is the risk of carbendazim residue exposure from vegetable consumption, CDI is the exposure of carbendazim residue from vegetable consumption per day (g day^{-1}), and ADI is acceptable daily intake of carbendazim (mg kg^{-1} bw day^{-1}).

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 is 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.

3. RESULTS AND DISCUSSION

3.1 Method Efficiency

The objective of the present work was to develop a sensitive method for determining carbendazim residue in vegetables. Initially, ethyl acetate was used to extract carbendazim from vegetables according to previous studies' experiments [17] and the extract was acidified with hydrochloric acid and sodium acetate. Clean-up of the extract (7.5 mL) was performed by passing through the SAX/PSA dual-layer cartridges and the eluate (20 μL) was injected onto HPLC column for analysis. Method efficiency was performed under different experimental conditions in order to obtain the optimal condition.

3.1.1 Effect of extraction sample weight

In this study, the samples weighed 5 and 10 g were evaluated. The results found no significant effect on the extraction recovery (5 g vs 10 g : 85±9 % vs 85±7 %). Therefore, 5 g of sample weight was chosen for the experiment.

3.1.2 Effect of extraction solvent volume

The influence of the solvent volume on the extraction recovery was measured by varying the volume of ethyl acetate from 10, 15, 20 and 25 mL, respectively. The extraction recovery increased when the volume of ethyl acetate was changed from 10 to 15 mL and then decreased when the volume of ethyl acetate was changed from 20 to 25 mL. Based on the experimental results, 15 mL of ethyl acetate was chosen.

3.1.3 Effect of washing solvent volume

SAX/PSA dual-layer cartridge was washed with different volumes of ethyl acetate at 5, 10, and 15 mL. It was found that the extraction recovery increased as the ethyl acetate volume increased from 5 to 10 mL, however it remained unchanged between 10 and 15 mL. As a result, 10 mL of the ethyl acetate for washing a SAX/PSA dual-layer cartridge was suitable for the experiment.

3.2 Calibration Curve, Reproducibility, Limit of Detection and Limit of Quantitation

Quality parameters of the method (i.e. linearity, detection limits, recovery and precision) were studied under conditions using methanol standard solution for spiking with a concentration ranging from 0.05 to

0.30 mg kg^{-1} (Table 1). The correlation coefficient was 0.999 and the equation of the calibration curve was y = 193.8x - 0.31. The typical chromatograms of vegetable sample blank, vegetable samples spiked with carbendazim at a concentration of 0.1 mg kg^{-1} and real vegetable sample are shown in Figure 2.

Table 1. Recovery and precision (RSD) of carbendazim from spiked vegetable samples.

Spiked levels (mg kg^{-1})	% Recovery±SD	Precision (% RSD)
0.050	92.6±5.8	6.3
0.075	94.1±7.0	7.5
0.100	94.5±2.8	3.0
0.200	96.0±2.0	2.1
0.300	95.6±2.6	2.7

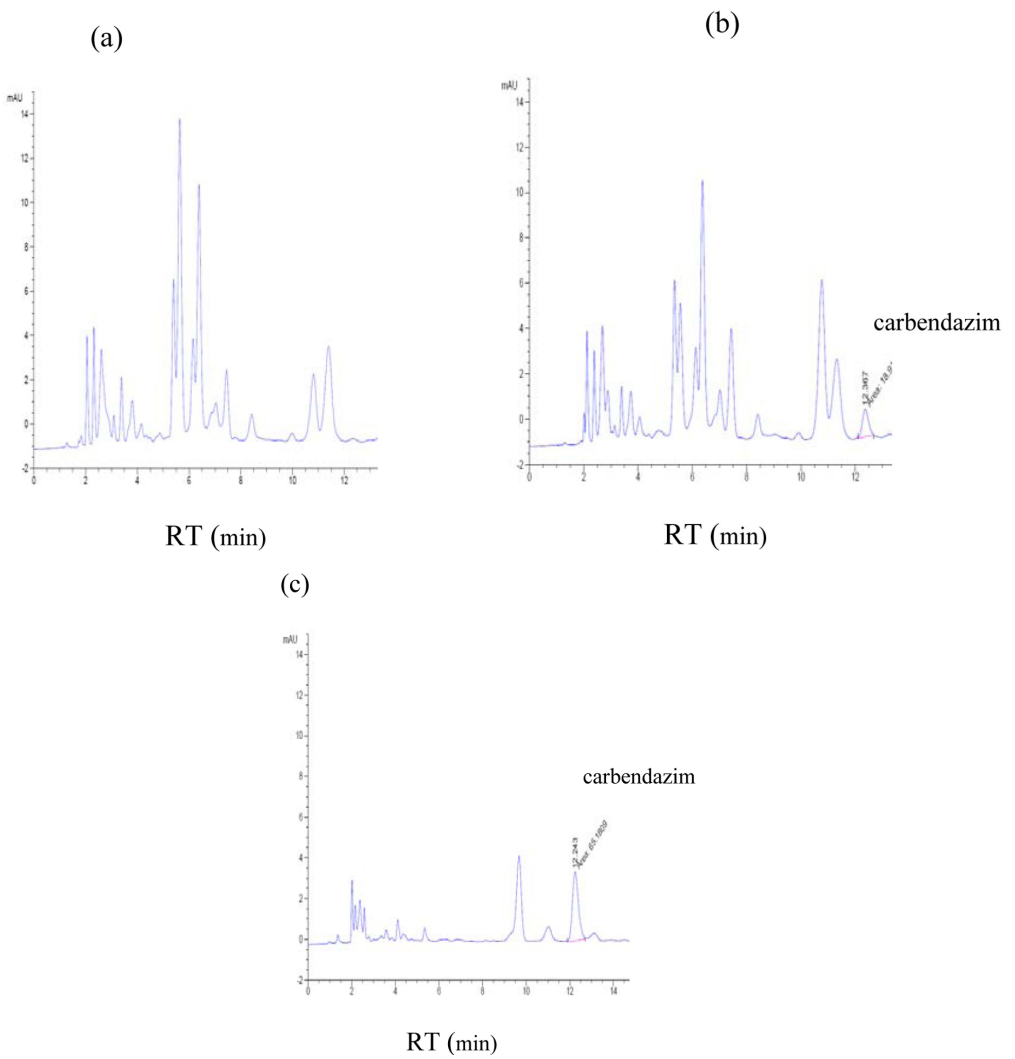

Figure 2. Chromatograms of (a) vegetable sample blank, (b) vegetable sample spiked with carbendazim at a concentration of 0.1 mg kg^{-1} and (c) an actual vegetable sample.

Recovery values were calculated as the ratio of the peak areas of the carbendazim from the spiked vegetable to the peak areas of standard solution. Recoveries were greater than 90% at 0.05-0.30 mg kg^{-1} spiking levels which is comparable with the report of Al-Ebaisat [10]. The LOD was computed from plotting the RSD versus concentration of spiked carbendazim and equation was y = 0.0056x + 0.003. Thus, the determined LOD value was 0.003 mg kg^{-1} and the LOQ was 0.03 mg kg^{-1}. The precision of quantitative measurements was checked at each concentration level of five levels by measuring five replications of standard solutions. In Table 1, the RSD of the precision test was lower than 10 % at each concentration.

3.3 Carbendazim Residues in Vegetable Samples from Chiang Mai City

The developed method was applied to a survey of carbendazim residues in vegetable samples. Eight kinds of common vegetables in Thai dish including Cauliflower (*Brassica oleracea* L. var. *botrytis* L.), ginger (*Zingiber officinale* Roscoe), kale (*Brassica albograba* Bailey), cucumber (*Cucumis sativa* L.), yard long bean (*Vigna sesquipedalis* Koprn), guinea-pepper (*Capsicum frutescens* L.), chili (*Capsicum annuum* L.), and tomato (*Lycopersicon esculentum* Mill.) were purchased from the four fresh markets in Chiang Mai city. Results showed in Table 2 that carbendazim was detected in 21 samples (66% of the total 32 samples) which is higher than the carbendazim residue detected in peaches and lower than in nectarines reported by Blasco *et al*. [22]. In addition, the present developed method showed that 5 g of vegetable sample was suitable for carbendazim residue determination.

Table 2. Carbendazim residue in 8 kinds of vegetable samples from four fresh markets in Chiang Mai city (August to October 2011).

Vegetable Samples (Scientific name)	No. of samples analyzed	No. of detected samples (%)	Residue level range (mg kg^{-1})	Mean±SD (mg kg^{-1})
Cauliflower (*Brassica oleracea* L.)	4	3 (75)	0.020-0.056	0.032±0.021
Chili (*Capsicum annuum* L.)	4	3 (75)	0.038-0.040	0.010±0.001
Cucumber (*Cucumis sativa* L.)	4	2 (50)	0.013-0.200	0.077±0.060
Ginger (*Zingiber officinale* Roscoe)	4	2 (50)	0.009-0.011	0.016±0.005
Guinea-pepper (*Capsicum frutescens* L.)	4	3 (75)	0.011-0.081	0.118±0.187
Kale (*Brassica albograba* Bailey)	4	2 (50)	0.034-0.119	0.036±0.039
Tomato (*Lycopersicon esculentum* Mill.)	4	3 (75)	0.014-0.064	0.039±0.001
Yard long bean (*Vigna sesquipedalis* Koprn)	4	3 (75)	0.008-0.334	0.035±0.026
Total	32	21**(66)**	0.008-0.334	0.045±0.043

3.4 Health Risk Assessment

The consumption of tomato, cucumber, kale, cauliflower and ginger was surveyed from people living in Suthep subdistrict, Chiang Mai city by specific randomizing sample areas and populations that covered all villages. A total of 244 participants, 100 males and 144 females, were enrolled including farmers, general employees, traders, office workers, government officers and business owners. The exposure assessment or CDI of carbendazim from tomato, cucumber, kale, cauliflower and ginger consumption was less than 100% ADI (0.03 mg kg^{-1}) as shown in Table 3. As the result of consumption of those 5 vegetables, there was no effect to the consumer health. The CDIs of carbendazim residue from consumption of tomato (64.1 %), cucumber (59.6 %), and kale (56.7 %) were greater than 50 % of ADI while of cauliflower (19.3 %), and ginger (0.9 %) were much lower though the commination may vary from season to season.

Table 3. Calculated daily intake (CDI) of carbendazim from 5 kinds of vegetables compared to ADI that set at 0.03 mg kg^{-1} bw day^{-1} (FAO/WHO).

Vegetable Samples (Scientific name)	CDI±SD (mg kg^{-1} bw day^{-1})	%ADI	CDI range (mg kg^{-1} bw day^{-1})
Tomato (*Lycopersicon esculentum* Mill.)	0.019±0.021	64.1	0.000-0.105
Cucumber (*Cucumis sativa* L.)	0.018±0.021	59.6	0.000-0.154
Kale (*Brassica albograba* Bailey)	0.017±0.016	56.7	0.000-0.090
Cauliflower (*Brassica oleracea* L.)	0.006±0.008	19.3	0.000-0.088
Ginger (*Zingiber officinale* Roscoe)	0.000±0.001	0.87	0.000-0.006

4. Conclusion

The present developed method showed to be sensitive for determining carbendazim residue in various kinds of vegetables with the LOD far below the Codex MRL. This method is also fast, simple with good recovery rate (92-96%) and employs only 5 g of sample for analysis. In addition, the sample preparation procedure was successfully applied in variety of vegetables without any problem encountered.

Regarding the health risk assessment from food consumption, assessment of pesticide exposure should be frequently conducted by using a sensitive and simple method. Thus, the study would be able to cover more kinds of vegetables and also fruits in order to serve the food safety policy of the society.

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