Chiang Mai J. Sci. 2007; 34(2) : 227-234
www.science.cmu.ac.th/journal-science/josci.html
Contributed Paper

Optimization Method for Determination of Carbofuran and Carboxin Residues in Cabbages by SPE and HPLC-UV

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Received: 29 December 2006

Accepted: 19 February 2007.

ABSTRACT

A study was undertaken to develop a method for carbofuran and carboxin residues analysis in cabbages (*Brassica oleracea* L. cv. white headed cabbage cruciferae) by HPLC equipped with UV detector. Cabbage samples were extracted with ethyl acetate followed by solid phase extraction (SPE) with florisil sorbent for cleaning up. The SPE condition was achieved by employing 3 ml of 70% acetonitrile/water as eluting solvent. The extracts were separated by reversed phase C_{18} column of HPLC-UV. The mobile phase used was 25% acetonitrile/water at 0.8 ml/min of the flow rate and detection wavelength at 205 nm. Linearity of the response was obtained with linear determination coefficients higher than 0.996. The lower limit of detection (LOD) value was in the range of 0.02-0.06 μg/ml. Percent recoveries for carbofuran and carboxin were 94.7% ± 0.7 and 90.0% ± 1.2, respectively. The method was employed for analysis of these two pesticide residues in 11 cabbage samples from various types of market. Five of those have safety labels and three of them are certified, while another six have no safety label. Carbofuran residue was not detected in any cabbage samples while the carboxin was found in three samples from both with and without safety label. Its residue was in the range of 0.054 to 0.414 mg/kg fresh weight. The samples were confirmed by HPLC-fluorescence and GC-MS, for carbofuran and carboxin respectively. The selected analytical methods gave agreeable result with each others. The carboxin, which was first detected by HPLC-UV in some of the extracted sample, was also found by GC-MS at the same level.

Keywords : carbofuran, carboxin, cabbage, HPLC, solid phase extraction.

1. INTRODUCTION

Pesticides are widely used to protect the crops from a variety of pests. The use of pesticides benefits in increasing agricultural production but the repeated and indiscriminated uses of certain pesticides have led to their accumulation in plants**,** animals, solid and sediments, thus effecting widespread contamination of the environment [1]. Such application of pesticides has the drawback of pesticide residues which remain on fruits and vegetables, constituting a potential risk to consumers. Fruits and vegetables are the foods that receive the highest doses of pesticides [2].

In this experiment, the widely used pesticides in agriculture protection carbofuran and carboxin were selected. Carbofuran

constitute a family of carbamate pesticides which cover a wide range of uses in the treatment of seed, soil and crops [3]. Carbofuran is in toxicity class I - highly toxic or toxicity class II - moderately toxic under the classification of EPA toxicity [4]. Carboxin is anilide fungicide and intensively applied at various stages of cultivation and during post harvest storage to provide protection against rotting [5]. Carboxin are in toxicity class III - slightly toxic [4]. Although it has low mammalian toxicity, fungicide residues levels in foodstuffs are generally legislated to minimize the exposure of consumers to the harmful or unnecessary intake of pesticides [5].

Most analytical methods for pesticide analysis are based on chromatographic techniques by both Gas Chromatography (GC) and High Performance Liquid Chromatography (HPLC). HPLC is obviously preferred approach for polar, less volatile and thermally labile pesticides such as carbamate. This is an effective method for separating and analyzing the various carbamate pesticides employing different detectors [6, 7]. The methods for the determination of pesticides residues in fruits and vegetables could employ reversed-phase chromatography with C_{18} or C_8 columns and aqueous mobile phase, followed by UV absorption, UV diode array detection, mass spectrometric or florescence detection [2].

Sample preparation is often the most time consuming step in a chemical analysis and the sample matrix frequently interferences with measurement. Solid phase extraction (SPE) is being increasingly used in food analysis, mainly for sample clean up. SPE clean up is inserted as a part of the chromatographic system, mostly using HPLC because of the compatibility of mobile phase [8]. SPE is similar to low pressure liquid chromatography.

The objective of this study is to study and develop a method for carbofuran and carboxin residues analysis by HPLC-UV and to determine their levels in cabbages (*Brassica oleracea* L. cv. white headed cabbage cruciferae).

2. MATERIALS AND METHODS

2.1 Reagents

Pesticide standards (carbofuran and carboxin) were ≥ 99% purity (ChemService, USA). HPLC grade acetonitrile (VWR Prolabo International Ltd., EC), methanol (Lab Scan, Ireland) and analytical grade of ethyl acetate (VWR Prolabo International Ltd., EC) were used. Deionized water was prepared with a Milli pore Q system. SPE columns (3 ml reservoir, 500 mg packing material) were from 2 companies. C_{18} SPE was from Waters, USA, while florisil SPE was from Supelco, Germany. For individual working standard solutions, 1 ml of each 1000 ppm stock standard solution was pipetted into a 10 ml volumetric flask and diluted to the mark with HPLC grade methanol to yield the standard solution of 100 μg/ml. Mixed standards 10 μg/ml was prepared by mixing 2.5 ml volumes of each 100 μg/ml working standard solutions into a 25 ml volumetric flask respectively and HPLC grade methanol was added to the mark. The working standard solutions were prepared by further dilution and stored at 4°C in refrigerator.

2.2 Optimization of High Performance Liquid Chromatography

High Performance Liquid Chromatography HP 1100 manufactured by Agilent, Germany consisting of Agilent 1100 series Isocratic pump and Agilent 1100 series variable wavelength detector was employed. Reversed phase liquid chromatography with 4.0 x 125 mm and 5 micron particle diameters of μBondapak C_{18} column was employed. The single standard solutions of 5 ppm of carbofuran and carboxin as well as mixed standards dissolved in methanol were measured for the maximum absorbance by UV-VIS spectrometer. The optimized HPLC mobile phase using the mentioned column was 25% acetronitrile in water (v/v) at a flow rate of 0.8 ml/min. Injection volume was 10 μl and detection wavelength was 205 nm. Total runtime was about 30 minutes.

2.3 Validation of the Method

A validation of the method was performed in terms of precision (reproducibility and repeatability), linearity range, limit of detection and limit of quantification. Precision of a method is the degree of scatter of the results and usually expressed as standard deviation (SD), and percent relative standard deviation of retention time and peak area. For testing of repeatability of HPLC system, 5 μg/ml mixed standards solution of carbofuran and carboxin was injected 10 times within intraday onto HPLC column under optimum conditions. In order to find the reproducibility, the same amount of standard solution was injected 3 days to observe reproducibility of retention times and peak area of the analytes from chromatograms. Linearity is assessed by calculating a linear regression coefficient (r^2). The linearity of the calibration curves was performed with 0.1, 0.2, 0.4, 0.6, 0.8, 1, 2, 4, 6, and 8 ¼g/ml of mixed pesticide standards. Series of mixed standards including 0.1, 0.2, 0.4, 0.6 and 0.8 ¼g/ml was injected into HPLC-UV to investigate the limit of detection (LOD) and limit of quantification (LOQ) [9].

2.4 Optimization of Solid Phase Extraction (SPE)

2.4.1 Study of SPE Separation and Elution Profile

The cartridges packed with C_{18} sorbent were loaded with three different concentration levels, 0.5, 1.0 and 2.0 ppm, of mix standard solutions after conditioning the bonded phase by 10 ml of methanol followed by 10 ml of Milli Q water. The composition and volume of the elution solvent were optimized. The composition of the eluting solvent was assessed by varying different concentrations of acetonitrile with water 40, 50, 60, 70 % using C_{18} SPE sorbent. In the elution step, the series of 1st fraction (3 ml), 2nd fraction (2 ml) and 3rd fraction (2 ml) of elutes were injected into HPLC-UV.

Elution volume of acetronitrile/ water (70:30) as the eluent for the analytes was optimized. Each pesticide at 2μg/ml fortification level was used to evaluate the eluent optimum volume. After passing through the SPE cartridges, 1 ml eluent was collected in each seven fraction.

2.4.2 Recovery Test

Sorbent selection is influenced by the analyte characteristics and sample matrix. To optimitize the efficiency of the SPE, C_{18} and florisil sorbents were tested. Reverse phase C_{18} and normal phase florisil sorbents of cartridges were studied. Recoveries were determined in triplicate at three concentration levels. The standard solutions were loaded after conditioning and recoveries were calculated from the chromatograms of the standard solutions before and after use of the cartridges.

2.5 Sampling Method

The popular and commonly consumed vegetables in Thai food namely cabbage (*Brassica oleracea* L. cv. white headed cabbage cruciferae) representing the Brassica families were selected for this study. Vegetable samples with and without safety labeled samples were collected from different markets such as major supermarket, minimart, farm shop and local fresh market of Chiang Mai, Thailand by using market oriented supply study. This kind of study monitors the marketed produce available to consumers by collecting samples from different shops [10]. Eleven cabbage samples were taken as the mentioned criteria. Among the samples, 5 of them are with safety label and 3 of them are certified, while another 6 are without safety label.

2.6 Sample Analysis

Ten grams of freshly chopped vegetable was homogenized and extracted with 50 ml of ethyl acetate and sonicated in the ultrasonic bath for 5 minutes. Thirty grams of anhydrous sodium sulphate were added and homogenized well for 10 minutes to remove water.

Then, the extract was filtered through the Buchner funnel containing Whatman filter paper No1. The 25 ml of ethyl acetate was added to the pellet to rinse for two times and then the rinsing liquid was added to the combined extraction fractions. The filtrate solution was evaporated in the rotary evaporator at 30 ºC until dryness and redissolved in 5 ml of 70 % acetronitrile / water. Only 2 ml of the redissolved extract was centrifuged. After that 0.5 ml of the supernatant was loaded onto florisil SPE cartridge which had already been conditioned with 10 ml of methanol and 10 ml of Milli Q water. The column cartridge was dried with vacuum manifold and 3 ml of 70% acetronitrile /water were used to elute the analytes retained on the florisil sorbent before injection onto the HPLC column.

2.7 Confirmation Method

It is important to confirm the identity of analytes and standards by comparing their retention time. Some of the samples were spiked with known mix standards and injected on the HPLC system under the same optimum condition. The chromatograms of with and without spiking of the standards in some extracted samples were compared. In addition, some of the extracted samples were analyzed by Laboratory Center for Food and Agricultural Products Co., Ltd (LCFA) by using HPLC fluorescence detector for carbofuran and GC-MS for carboxin respectively.

3. RESULTS AND DISCUSSION

3.1 HPLC- UV Chromatographic Conditions

HPLC condition was developed by employing 25% acetronitrile/water of mobile phase at a flow rate of 0.8 ml/min detected by 205 nm UV detector. Chromatogram of mixed standards under the optimum conditions is shown in Figure 1.

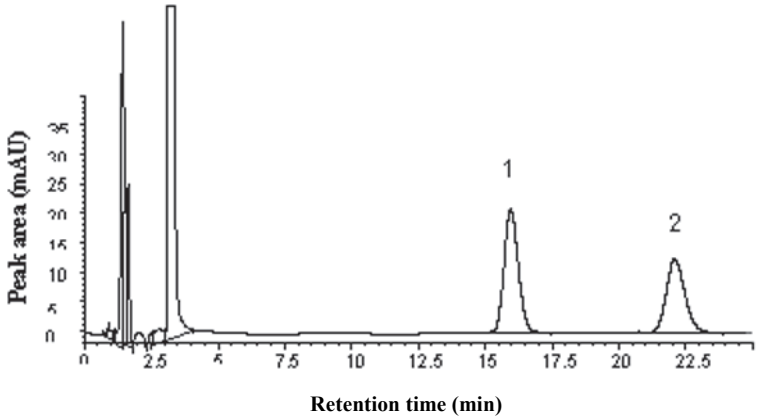

Figure 1. HPLC Chromatograms of 8 μg/ml mixed standards obtained under the optimized condition. Peak no.1 = Carbofuran, no. 2 = Carboxin.

3.2 Validation of the Method

All of the parameters were needed as a part of an evaluation of the instrument efficiency. Satisfactory reproducibility precision (n = 3) in terms of % RSD of the retention time and peak area was in a range of 0.61 to 2.83% and 0.02 to 0.67% respectively. The repeatability precision values (n = 10) in terms of % RSD of the retention time and peak area was in the range of 0.12 to 0.22% and 2.24 to 2.7%. Good linearity of the response was found for all pesticides with linear determination coefficients higher than 0.9962. The LOD values of carboxin and carbofuran were 0.02 and 0.06 μg/ml, respectively, while the LOQ values of those were 0.08 and 0.18 μg/ml, respectively.

3.2 Sample Clean Up by SPE

3.3.1 Separation and Elution Profiles

In the step of the SPE, C_{18} sorbent and florisil sorbent were compared at different levels of pesticide concentrations to evaluate the efficiency of the SPE Sep-Pak. The characteristic and concentration of the analytes as well as the sample matrix affected to the retained capacity of the SPE were studied. A separation profile and an elution profile of analytes are shown in Figures 2 and 3, respectively.

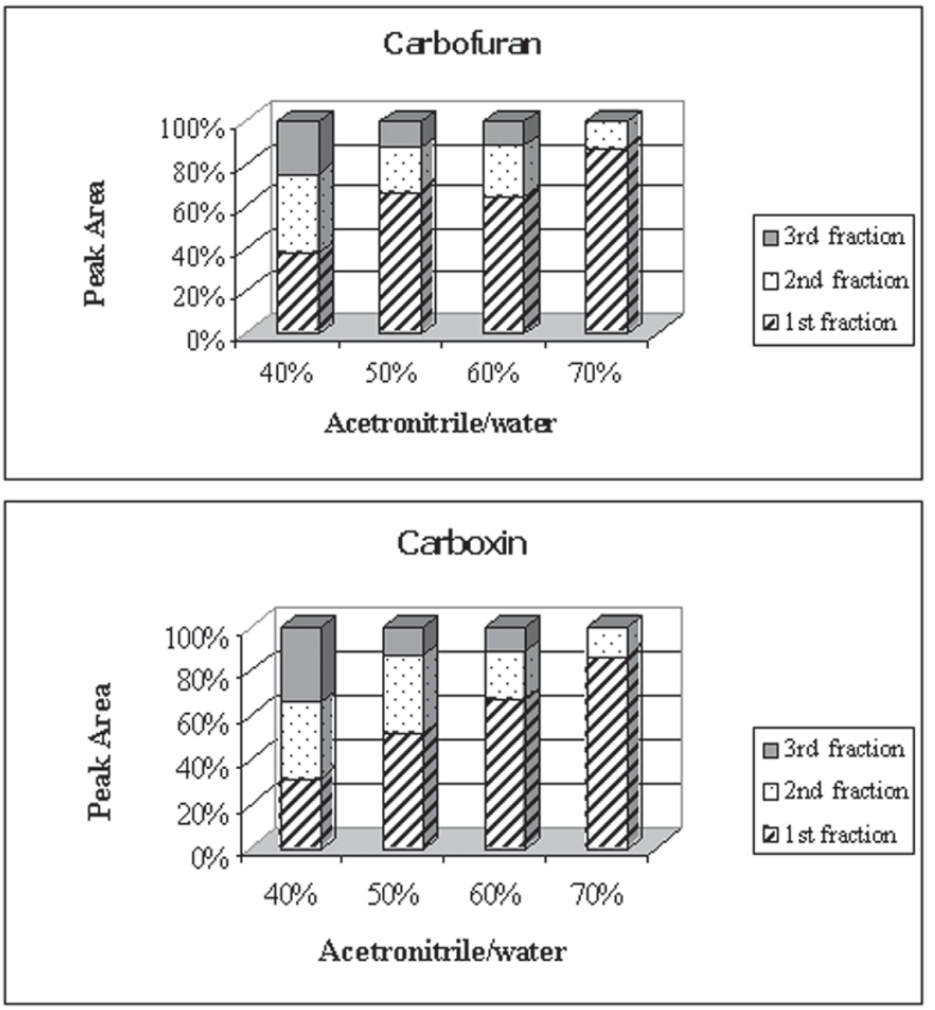

Figure 2. Separation profiles of analytes obtained from C_{18} SPE at different concentrations of acetonitrile/water.

The total peak area obtained by different composition of acetonitrile in water was not significantly different from one another. The highest peak area was found in first fraction and decreased from second to third fraction. The eluting solvent of 40%, 50%, and 60% acetonitrile in water showed similar patterns with all analytes in each fraction. 70% acetonitrile in water eluted all of the analytes in 1st and 2nd fractions. The eluting strength of 70% acetonitrile solvent is sufficiently enough to elute all analytes in the first two fractions and no peak was found in 3rd fraction (Figure 2). Consequently, 70% acetonitrile in water was chosen as the eluting solvent for the extraction of carbofuran and carboxin by

using C_{18} SPE sorbent.

The elution profile of 70% acetonitrile eluting solvent was drawn from 1 ml eluting series. Figure 3 shows that most of carbofuran and carboxin were found until the fraction no. 2. Only 3% of carboxin was found in the 3rd fraction. Thus, the volume of 3 ml was chosen to be the optimum eluent volume.

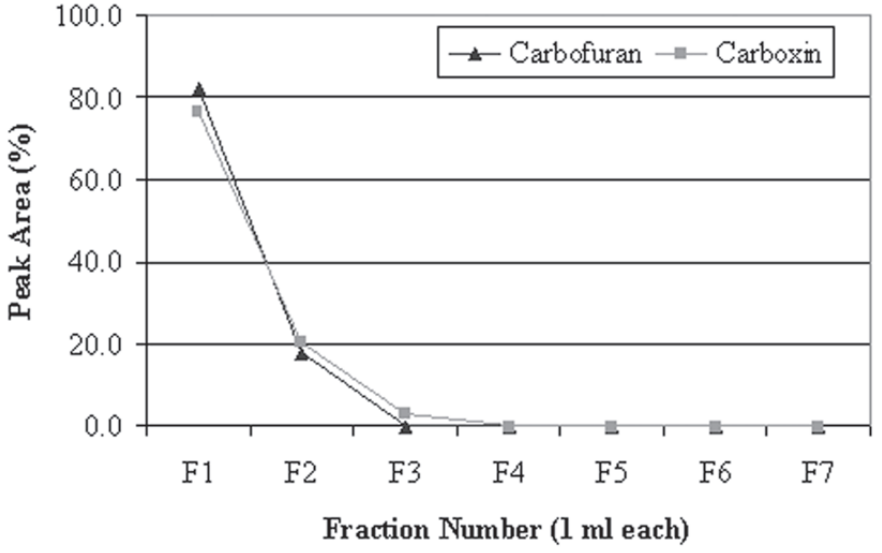

Figure 3. Elution profiles of carbofuran and carboxin obtained from C_{18} SPE eluted with 70% acetonitrile/water at 2 μg/ml fortification level.

3.3.2Recoveries of the Analytes

The sample was extracted by the optimized extraction procedure. The recovery rate of each pesticide at 0.5 μg/ml fortication level was evaluated in order to access the extraction efficiency of the proposed method. There was much interference in the recovery studies of the real samples. The sample contains co-extractives and co-eluted with the interest of pesticides. Relatively high background was encountered for the peak identification and quantitation and resulted in poor recoveries. Percent recoveries of analytes obtained from different SPE sorbents are illustrated in Table 1. It was found that normal phase florisil SPE resulted in the higher recoveries than the reverse phase C_{18} for all concentrations of the analytes. Florisil SPE cartridges resulted adequate recoveries (87.1% – 106.4%) for all pesticides at 0.5 and 1.0 ppm fortification levels. C_{18} resulted adequate recoveries only at 0.5 ppm fortification level but low recoveries were obtained at higher concentration levels of 1.0 ppm and 2.0 ppm. Consequently, florisil sorbent was selected for the SPE clean up.

Table 1. Percent recoveries of analytes using different SPE sorbents.

Pesticides	% Recovery ± SD					
	0.5 ppm		1.0 ppm		2.0 ppm	
	Florisil	C_{18}	Florisil	C_{18}	Florisil	C_{18}
Carbofuran	101.8± 2.0	97.8± 0.9	99.6± 2.3	71.2± 3.3	57.4± 1.6	36.4± 1.3
Carboxin	106.4± 2.9	93.5± 1.6	87.1± 3.2	68.9± 0.9	60.3± 3.2	38.6± 0.5

3.4 Analysis of Samples

Cabbage samples were extracted and analysed using florisil SPE followed by HPLC-UV as the methods mentioned in topic 2.5. Pesticide residues were detected in some of the extracted samples and therefore the standard of suspected pesticide was spiked into those extracts and analysed again by HPLC-UV.

The proposed method has been applied in the analysis of cabbage samples. The samples were pretreated with the optimum extraction. Level of concentrations and frequency of carboxin residues found in cabbage samples are shown in Table 2.

Table 2. Amount of carbofuran and carboxin detected in cabbage samples (n = 3).

No.	Sample code	Sample Description	Amount ± SD (mg/kg)	
			Carbofuran	Carboxin
1	LF1	Local fresh market (without safety label)	ND	ND
2	SM1	Super market (without safety label)	ND	ND
3	SM3	Super market (*with certified* safety label)	ND	ND
4	MM1	Minimart (without safety label)	ND	ND
5	FS1	Farm shop (with safety label)	ND	ND
6	LF2	Local fresh market (without safety label)	ND	ND
7	SM2	Super market (without safety label)	ND	0.414 ± 0.074
8	SM4	Super market (*with certified* safety label)	ND	ND
9	MM2	Minimart (without safety label)	ND	0.065 ± 0.005
10	MM3	Minimart (*with certified* safety label)	ND	0.054 ± 0.014
11	FS2	Farm shop (with safety label)	ND	ND

Carbofuran was not detected in any samples. The amounts of carboxin found in cabbage samples were in the range from 0.054 to 0.414 mg/kg fresh weight. The concentration was calculated from concentration in the solution (μg/ml) multiply with the extract final volume and dilution factor (10 times) and divided by fresh weight of cabbage sample.

The residues in the sample with the safety label are likely to be at lower level than those in the sample without safety label. 20 % of analyzed samples with safety label and 33 % of those without safety label gave positive values.

3.8 Confirmation for the Analyte

Confirmation method was achieved on the basis of the comparison of the spiking the standards at 0.1 ppm fortification level (retention time = 22.2 min and peak area = 5.80 mAU) in the selected extracted sample (SM2; supermarket without safety label) under the same optimum condition of HPLC-UV. The spiked chromatogram shows higher peak area of carboxin at the similar level of the carboxin standard added. It can be concluded that the detected peak at retention time equals to 22 min in the SM2 sample was the carboxin.

In addition of spiking the standard to the sample, some samples were also confirmed by the Laboratory Center for Food and Agricultural Products Co., Ltd (LCFA) in Chiang Mai. In MM2 sample (Minimart without safety label) carbofuran was not detected by HPLC equipped with fluorescence detector, whereas carboxin was detected by GC-MS. The retention times of the carboxin standard and the analyte found

in GC-MS were 20.11 and 20.17 minutes, respectively. Moreover, comparison data of the same analyte obtained from the same sample using two different techniques (HPLC-UV and GC-MS) gave agreeable value of detected carboxin (0.02 μg/ml) in the MM2 sample.

4. CONCLUSIONS

The extraction of carbofuran and carboxin in cabbage samples using florisil sorbent solid phase extraction following with HPLC-UV analysis has been demonstrated as a reliable analytical tool for the residues analysis. In order to compensate the matrix effect, the amount and concentration of sample matrix in the loading step of SPE have to be strongly considered. Large amount and concentrated sample matrix affected the retained capacity of the SPE to an extent resulting in a matrix enhancement effects. Therefore, it was proposed that, smaller amount of extract to be used for the clean up. In addition, centrifugation was essentially help to settle down the solid matter before passing the SPE.

No residue of carbofuran was detected in any of 11 cabbage samples whereas low level of carboxin residues was detected in only 3 samples in the range from 0.054 to 0.414 mg/kg fresh weight. The residues found in the sample with the safety label are likely to be at lower level than those in the sample without safety label. In summary, 20 % of the analyzed samples with safety label and 33 % of them without safety label gave positive values of carboxin.

ACKLOWDGEMENTS

The authors thank the Thai International Development of Coorporation Agency (TICA) for funding of this research work.

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