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CARBENDAZIM RESIDUES QUANTIFICATION IN GREEN LETTUCE BY ENZYME-LINKED IMMUNOSORBENT ASSAY

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Abstract: *Carbendazim is a fungicide that is not approved for use in the European Union, but is used in some other countries to preserve agricultural crops. Enzyme-linked immunosorbent assay (ELISA) become a viable alternative to traditional instrumental methods in pesticides analysis, for screening purpose. Carbendazim residues were determined in two green lettuce cultivars, from the experimental lots, harvested at different times after the treatment with 0.1 % carbendazim solution, by an ELISA carbendazim commercial kit and the values were compared with those obtained by high performance liquid chromatography (HPLC). The results of ELISA and HPLC analysis of carbendazim residues in the two green lettuce cultivars were well correlated (Pearson correlation coefficient r=0.8925). Carbendazim residues in green lettuce/vegetables can be determined with high reliability by commercial ELISAs with results well correlated with those obtained by HPLC, with an additional sample preparation step involving solid phase extraction, in order to reduce matrix effects; the assay can be used for carbendazim screening of many vegetable/fruit samples in shorter time and with inexpensive/cheaper equipment.*

Keywords: *carbendazim, green lettuce, ELISA, HPLC.*

1. Introduction

The concern about the food safety with reference to pesticide residue levels and the appropriate analysis methods is constant [1-3]. Carbendazim (Metil N-benzimidazol-2-carbamat) belongs to carbamate pesticide class and is a fungicide used in fruit and vegetable growing and viticulture. This pesticide is not approved for the use in the European Union [4], but it is used in some other countries to preserve agricultural crops. Carbendazim causes embryotoxicity, apoptosis, teratogenicity, infertility, hepatocellular dysfunction, endocrine disrupting effects, disruption of haematological functions, mitotic spindle abnormalities mutagenic and aneugenic effects [5]. Carbendazim residues are detected by high performance liquid chromatography (HPLC) with UV [6,7], fluorescence detector [8] or by liquid chromatography-mass spectrometry [9]. But simple, high-throughput and fast analytical techniques with low cost are in great need as an alternative to traditional instrumental methods for screening purposes [10]. And so immunoassays become a viable alternative to traditional instrumental methods, especially enzyme-linked immunosorbent assay (ELISA), characterized by increased specificity and

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sensitivity, simple operation, the possibility of analyzing a large number of samples in a short time and low costs per analysis. Itak et al. [11] developed a competitive ELISA for the quantitation of benomyl (as carbendazim) and carbendazim in water. This immunoassay can also be used for the determination of carbendazim in fruit juice. Williams et al. [12] reported that some commercial ELISA kits designed to detect pesticide residues in water can, with care and experience, be successfully applied as a screening technique for residues in food extracts. A modified enzyme immunoassay method has been developed to determine methyl 2-benzimidazole carbamate in fruit juices and concentrates and the agreement between this method and high-performance liquid chromatography was good [13].

The aim of our study was to determine carbendazim residues in two green lettuce cultivars, from the experimental lots, harvested at different times after the treatment with 0.1% carbendazim solution, by an ELISA carbendazim commercial kit and to compare the results with values obtained by high performance liquid chromatography.

2. Materials and method

2.1. Field trial

The experience was conducted into the Department of Horticultural Cultures in Protected Areas, HORTING Institute, Bucharest, Romania, during the vegetation period of the vegetable plants grown in a block type cold greenhouse. The biological material used was represented by two green lettuce cultivars: Allegiance and Lollo Bionda, intended for fresh consumption. The treatment with 0.1% carbendazim solution (prepared in the chemistry lab) was achieved by sprinkling the plants during the vegetation period. The samples were collected at different intervals after carbendazim treatment (3; 8; 13 days) and kept at -20^{0}C before being analysed.

2.2. Reagents and standards

MaxSignal® Carbendazim ELISA Test Kit was purchased from Bioo Scientific (Austin, Texas, USA). Carbendazim (97 %) was purchased from Sigma-Aldrich (Darmstadt, Germany). A carbendazim stock solution (250 µg/ml) was prepared in methanol and used for the preparation of working standard solutions necessary for calibration curve (1; 2.5; 5; 7.5; 10; 12.5 and 15 µg/ml) in HPLC. All other reagents used were p.a. grade and solvents were HPLC grade.

2.3. Analytical procedure

Analysis of carbendazim by ELISA

MaxSignal® Carbendazim ELISA Test Kit is a competitive enzyme immunoassay for the quantitative analysis of carbendazim in honey, juice, meat, rice/feed. Vegetables are complex matrices, so a response enhancement could appear in ELISA. For this reason we first clean up the vegetables extracts by solid phase extraction. Samples were well blended and 10 g sample were extracted in 20 ml methanol. The extracts were filtered through Whatman No. 1 filter papers (GE Healthcare Life Sciences Whatman); the filtrates were cleanup on OASIS MCX cartridges (Waters, Ireland) following the manufacturer instructions, then were concentrated to 1 ml using a TurboVap LV equipment (Caliper LifeSciences, USA). The concentrated filtrates were prediluted 1:100 with phosphate-buffered saline (pH 7.2), then to 1:500 with sample extraction buffer F, and after used in ELISA according to the protocol described by kit manufacturer. Samples and calibration standards were analyzed in duplicate. Absorbance was recorded on a microplate reader EZ Read 400 Research (Biochrom, Massachusetts, USA) with a 450 nm filter.

Analysis of carbendazim by HPLC

Samples were well blended and 10 g sample were extracted in 20 ml methanol. The extracts were filtered through Whatman No. 1 filter papers (GE Healthcare Life Sciences Whatman); the filtrates were cleanup on OASIS MCX cartridges (Waters, Ireland) following the manufacturer instructions, then were concentrated using a TurboVap LV equipment (Caliper LifeSciences, USA), so that the injected volume contained an amount of carbendazim within the linear range of the diode array detector. Finally the samples were filtered through 0.45 µm syringe filters (Thermo Scientific, USA) prior to injection. The chromatographic separation was performed using a LichroCART Purospher RP-18 column (250 * 4 mm), with 5 µ particle size (Merck KGaA, Germany) and the mobile phase consisted of water and methanol (25:75 - by volume) under isocratic chromatographic conditions, with a flow rate of 1 mL/min. The column temperature was set at 20^0C. Carbendazim was detected at 286 nm by the diode array detector [14]. Samples were analyzed in duplicate.The data acquisition and processing have been done with the ChromQuest 4.2. software (Thermo Electron Corporation, USA).

We calculated the average, standard deviation, and then calculated the Pearson correlation coefficient (r) to express the correlation between the concentration of carbendazim by ELISA and HPLC. The value of r ranges from -1 to +1. The closer |r| is to 1, the stronger is the correlation between the variables [15]. The results were statistically processed using GraphPad Prism (version 8.0.2, GraphPad Software Inc., San Diego, CA) [*16*].

3. Results and Discussion

In ELISA the standard calibration curve was prepared in the concentration range of 0.5 to 12.0 ng/ml (0.5; 1.5; 3.0; 6.0; 12.0 ng/ml) and the correlation coefficient was 0.9982 (Fig. 1). An enzyme-linked immunosorbent assay kit was used for the analysis of carbendazim residues in fruit and vegetables [17]. The authors observed a response enhancement in ELISA due to the sample matrix and controlled this issue using a strawberry sample extract free of carbendazim for standard preparation. In this study we attenuated interferences in ELISA response by cleaning up the vegetables extracts using solid phase extraction.

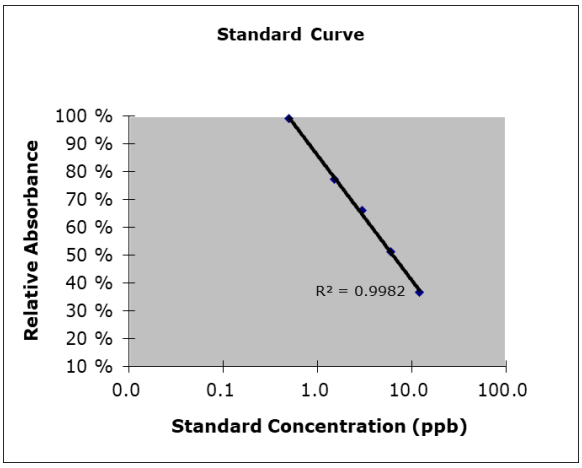

Fig. 1. MaxSignal Carbendazim ELISA Test Kit standard curve

Carbendazim residues were detected (at concentrations >0.001 mg/kg) in 8.3% of 20469 vegetable samples from 31 Chinese provinces, analyzed between 2014 and 2016 [18]. Carbendazim was found most often in cowpeas, celery, beans with pods, lettuces, cucumbers, and leeks. Lettuces contained the highest mean carbendazim concentration.

Carbendazim residues in green lettuce samples collected at different post treatment days are presented in Table 1.

For both cultivars carbendazim residues in samples collected on 3, 8 respectively 13 days after treatment with 0.1% carbendazim solution were between 0.21 and 3.05 mg/kg. The results of ELISA and HPLC analysis of carbendazim residues in

the two green lettuce cultivars were well correlated (r=0.8925) (Fig. 2).

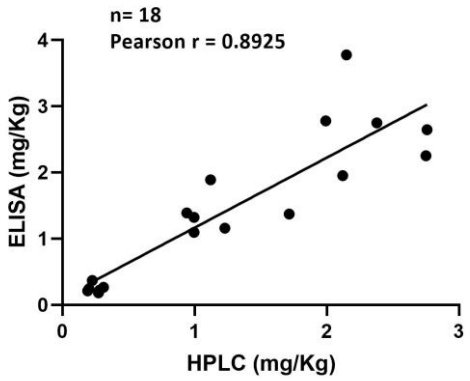

Fig. 2. Correlation between carbendazim concentrations as determined by ELISA and HPLC in green lettuce (n=18, Pearson correlation coefficient r=0.8925)

Mountfort et al. [17] reported a correlation coefficient of 0.91 between ELISA and HPLC for the analysis of carbendazim residues in fruit and vegetables.

Our results for both cultivars on 3 and 8 days are similar with those obtained by Bhattacherjee et al. [19] in mango fruits pulp after pre-harvest application of carbendazim at the rate of 0.1% (2.24 mg/kg on 4 days, respectively 1.23 mg/kg on 9 days), but the values obtained on 13 days were lower (0.51 mg/kg on 15 days in mango fruits pulp at the rate of 0.1%).

Table 1.
Carbendazim residues in Allegiance and Lollo Bionda cultivars determined by ELISA and HPLC

	Carbendazim residues (mg/kg)±SD (n=3 samples/cultivar/test)					
	Time interval post carbendazim treatment					
	3 days		8 days		13 days	
Cultivar	**ELISA**	**HPLC**	**ELISA**	**HPLC**	**ELISA**	**HPLC**
Allegiance	3.05±0.62	2.43±0.31	1.21±0.15	1.05±0.15	0.21±0.03	0.22±0.04
Lollo Bionda	2.33±0.42	2.29±0.40	1.53±0.31	1.28±0.38	0.29±0.07	0.27±0.04

SD=standard deviation

4. Conclusion

Carbendazim residues in green lettuce/vegetables can be determined by MaxSignal Carbendazim ELISA Test Kit, with an additional sample preparation step involving solid phase extraction. The results of ELISA and HPLC analysis of carbendazim residues in the two green lettuce cultivars correlated well (Pearson correlation coefficient r=0.8925). In addition, ELISA technique implies shorter analysis time than chromatographic method, that is very important for screening of a large number of fresh vegetables/fruits that are perishable. ELISA also implies smaller solvent volumes, less expensive and sophisticated equipment.

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All the authors declare no conflict of interest.

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