Determination of Benomyl Residues in Shiitake Mushrooms (*Lentinula edodes*) by Liquid Chromatography with UV Detection

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Abstract

A method is optimized to determine benomyl (as carbendazim derivative) in shiitake mushrooms. It is based on the extraction of the fungicide with an ethyl acetate–hexane mixture and a further analysis of the extract by high-performance liquid chromatography–UV. Mean recoveries are evaluated and range from 76% to 86% with relative standard deviation between 1.1% and 5.8%. The limit of quantitation (0.5 mg/Kg) is lower than the maximum residue level established by European legislation. The method is successfully applied to the analysis of shiitake mushrooms cultivated on eucalyptus logs treated with Benlate 500 (benomyl as active ingredient) under natural environmental conditions.

Introduction

The shiitake mushroom (*Lentinula edodes*) is one of the most common edible mushrooms in Brazil. The traditional production system utilizes natural oak or eucalyptus logs. However, edible mushrooms are characterized by a short shelf-life (1–3 days at room temperature) linked to the occurrence of postharvest changes. These changes are due to the high moisture content and enzymatic activity. Thus, the drying process is by far the most widely used method for guaranteeing long-term storage. On the other hand, mushrooms are susceptible to pest attacks and diseases during their growth on natural logs throughout the year. Therefore, a range of pesticides is available for both prophylatic and remedial use. For example, pesticides such as chlorpyrifos, cypermethrin, cyromazine, deltamethrin, dichlorvos, diflubenzuron, methoprene, permethrin, pirimiphos–methyl, prochloraz, and thiopanate–methyl have been used to control opportunist fungi like *Verticillium fungicola*, *Dactylium dendioides*, and *Mycogone perniciosa* (1), whose presence make mushroom commercial exploitation impracticable. A surveillance monitoring for pesticides in mushrooms from wholesale cooperatives in Northern Ireland found that 62% of mushroom samples contained detectable residues of prochloraz, permethrin, carbendazim, dichlorvos, resmethrin, γ-HCH, methoprene, chlorothalonil, malathion, diflubenzuron, and diazinon. For this reason, monitoring pesticide residues could aid in the optimization of shiitake production and its commercial exploitation (2).

Benomyl, methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate), is stable under strongly acidic conditions, but degrades rapidly in chemical (organic solvents and middle acidic aqueous solutions) and biological systems (animals, soil/sediments, and plants) through the loss of the *n*-butylcarbamoyl group to give carbendazim (3). The molecular structures of benomyl and carbendazim are shown in Figure 1.

Only a few methods have been reported to determine pesticide residues in a mushroom matrix. In these methods, ben-

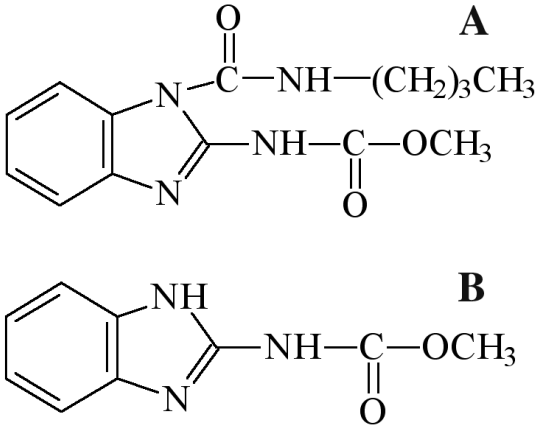

Figure 1. Molecular structures of benomyl (A) and carbendazim (B).

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zoylphenylurea insecticides (diflubenzuron, triflumuron, telubenzuron, hexaflumuron, lufenuron, flufenoxuron, and chlorfluazuron) were extracted by using acetone and partitioned into dichloromethane–cyclohexane (4) or dichloromethane–petroleum ether (5) and cleaned up by size-exclusion chromatography (4) or solid-phase extraction on aminopropyl-bonded silica cartridges (5). Analyses have been carried out by liquid chromatography (LC) with diode array and/or mass spectrometric (MS) detection (4,5). Another method is based on acetone–petroleum ether extraction and gas chromatography (GC) with electron-capture detection to determine allethrin residues in mushrooms (6). On the other hand, several reports are available in the literature about the determination of benomyl residues in other vegetable and fruit matrices (7–12).

The main objective of this work was to investigate the performance of a method for the determination of benomyl residues, as carbendazim derivative, in shiitake mushroom samples. This method involves a small-scale extraction, conversion of benomyl residues to carbendazim, and quantitation by LC using UV detection.

Experimental

Chemicals, reagents, and standards

Ethyl acetate, methanol, and hexane were nanograde (Mallinckrodt Baker, Paris, Kentucky). Sodium hydroxide was analytical grade (Mallinckrodt Baker). Benlate 500 (500 g/Kg) of benomyl was purchased by DuPont Produtos Agrícolas Ltda (São Paulo, Brazil). The high-performance liquid chromatography (HPLC) grade water was obtained by filtering deionized water through a 0.45-µm filter with a Waters Millipore (Milford, MA) system under vacuum. Methanol and water were degassed using a Branson 5200 (Branson Ultrasonic, Danbury, CT) ultrasonic bath.

A certified standard of carbendazim was purchased from Dr. Ehrenstorfer (Augsburg, Germany). The standard was 97.0% pure. Methanol was used to prepare the stock and working solutions according to appropriate dilutions. All solutions were stored at –18°C.

Apparatus

A Waters LC equipped with two solvent delivery pumps (Model 501), an injector (Model U6K), a UV–vis absorbance detector (Model 486), and a reporting integrator (Model 746) was used for the determination of carbendazim. A stainless steel analytical column LiChrospher 100 RP_{18} (125 × 4.0 mm i.d., 5 µm, Merck, Darmstadt, Germany) connected to a LiChrospher 100 RP_{18} guard column (20 × 4.0 mm i.d., 5 µm, Merck) was used. The compound was analyzed by an isocratic mode of methanol–water (7:3, v/v) at a flow rate of 0.8 mL/min using UV absorption at 286 nm.

Cultivation of shiitake mushroom and treatment of eucalyptus logs with Benlate 500

Shiitake mushrooms (*Lentinula edodes*) were cultivated on sawdust-based cultures at the Laboratório de Biologia e Zootecnia of Universidade Estadual Paulista (Unesp, Ilha Solteira, São Paulo, Brazil). Eucalyptus logs (*Eucalyptus urophylla*), approximately 1.0-m long and 10-cm in diameter were inoculated with sawdust containing one strain of shiitake mushroom. After completion of the spawn run (6 months) the colonized logs were set to produce shiitake.

Eucalyptus logs were treated with an aqueous suspensions of the commercial formulation Benlate 500 (500 g/Kg of benomyl, DuPont Produtos Agrícolas Ltda) using a costal sprayer. Each set of five logs was treated with a different dose of the formulation: 10, 50, and 100 µg/mL, respectively. After application, sampling was performed by randomly collecting from various parts of the experimental plots. Three shiitake mushrooms were taken from each log. Further, the shiitake mushrooms were divided into two sub-samples. The part of the shiitake sample submitted to the Benlate 500 treatment was packed in pored polyethylene film bags, sealed, and stored in a freezer (–18°C), and the other portion was oven dried (55°C/5 h), placed in polyethylene film bags, sealed, and stored in a freezer (–18°C). A separate plot of mushrooms was used to obtain untreated shiitakes for assessing the accuracy of the method developed for residue detection. Each replicate of fresh, dehydrated, and controlled shiitake was delivered to the laboratory for extraction and analysis.

Sample preparation and recovery assays

Shiitake mushroom samples (in natura and dehydrated) were triturated using a household blender and stored in closed glass flasks at –18°C.

For the recovery tests, fortified shiitake were prepared by adding a 500-µL aliquot of carbendazim standard solutions (2.5–50 µg/mL) to 5 g of untreated shiitake (in natura and dehydrated). The samples were allowed to equilibrate for 30 min prior to the extraction procedure.

Method

A 5 g portion shiitake mushroom sample was weighed into a glass-stoppered flask. A 10.0-mL aliquot of hexane–ethyl acetate mixture (2:3, v/v) was added, and the flask (approximately 50 mL) was shaken for 30 min on a mechanical shaker (Thermolyne, Dubuque, IA) at room temperature. A 5.0-mL aliquot of organic phase was extracted by using 2.5 mL of hydrochloric acid (1 mol/L). The aqueous phase was neutralized by using 5.0 mL of sodium hydroxide (0.5 mol/L) and extracted with 5.0 mL of ethyl acetate. The extract was evaporated in rotary evaporator (40°C) until 1.0 mL and dried under an N_2 stream. The residue was reconstituted to a 1.0 mL dose by a mobile phase. An aliquot of 20 µL was injected into the HPLC-UV system.

Results and Discussion

Method validation

A previous LC method based on a general extraction procedure using hexane–ethyl acetate mixture (1:1, v/v) in the presence of anhydrous sodium sulphate for the determination of carbendazim in grapes (13) was optimized for the determination of this fungicide in shiitake mushroom treated with Benlate 500.

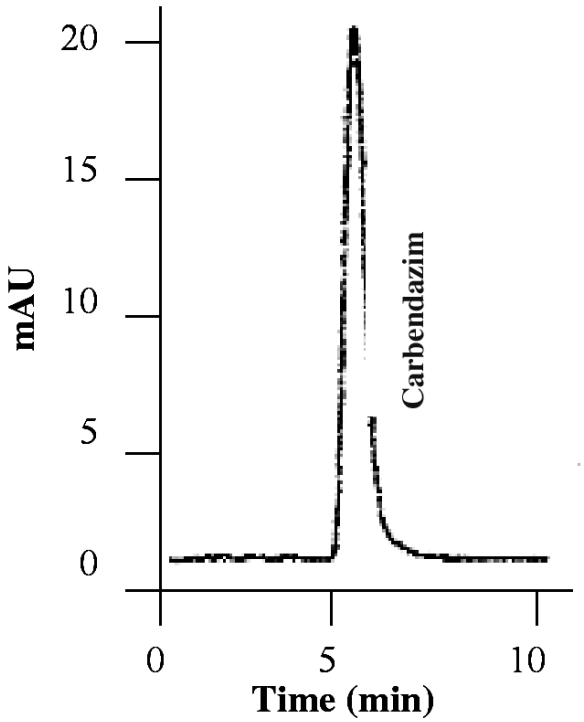

Figure 2. HPLC–UV chromatogram of standard solution of carbendazim at 2 µg/mL. Retention time of the carbendazim peak is 5.4 min. For chromatographic conditions, see the Experimental section.

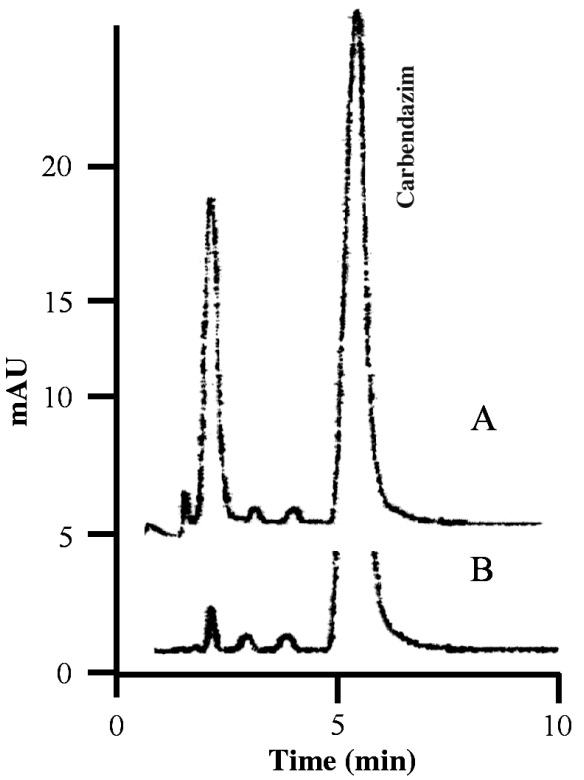

Figure 3. HPLC–UV chromatograms of dehydrated shiitake fortified at 2.0 µg/mL (A) and carbendazim standard solution at 2.0 µg/mL (B). Retention time of the carbendazim peak is 5.4 min. For chromatographic conditions, see the Experimental section.

Considering that benomyl standard solutions in all solvents, including water, always show some degree of conversion to carbendazim, a correction had to be made (14). Taking this fact into account, a procedure for the conversion of benomyl into carbendazim by treating it with acid solution has been optimized. The acidic layer was neutralized with sodium hydroxide, and the compound of interest was extracted from the aqueous solution by partitioning with a hexane–ethyl acetate (2:3, v/v) mixture. This mixture of solvents was found to be the most suitable for carbendazim extraction from shiitake mushroom.

LC analyses of carbendazim were performed on octadecyl reversed-phase. It was found that methanol–water isocratic elution (7:3, v/v) as the mobile phase at 0.8 mL/min and the detection at 286 nm showed to be the best condition with regards to the pesticide analyses. Figures 2 to 4 show chromatograms of the standard solution of carbendazim at 2.0 µg/mL, with the shiitake sample fortified by carbendazim at 2.0 µg/mL and the shiitake control sample (in natura and dehydrated), respectively. There were few peaks of the endogenous compounds in the control shiitake chromatograms, but the retention times were not matched by those of the fungicide peak. Therefore, they did not interfere with the determination of the carbendazim. The identification of the fungicide was realized by the retention time obtained when a standard solution of this pesticide was injected into the LC. Under these chromatographic conditions, the retention time for carbendazim was 5.4 min, and the total running time of HPLC-UV analysis was 10 min.

A study of recovery for carbendazim at four different fortification levels (0.5, 1.0, 2.0, and 10.0 mg/Kg) was carried out in order to assess the extraction efficiency of the proposed method. For

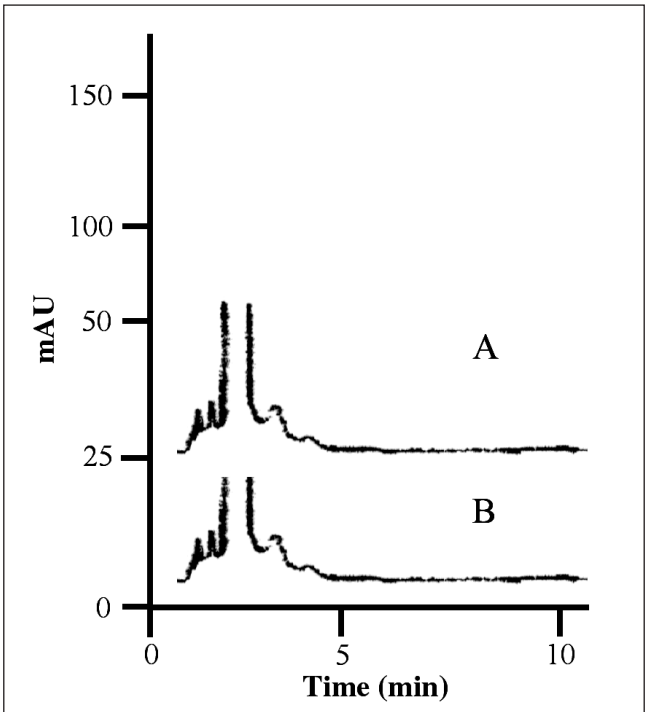

Figure 4. HPLC–UV chromatogram of: control sample of shiitake in natura (A) and control sample of shiitake dehydrated (B). For chromatographic conditions, see the Experimental section.

that, uncontaminated shiitake samples were spiked with the pesticide and processed as described. The quantitation of samples was based on peak areas obtained from injections of the shiitake extracts compared with those of the standards, and it was performed using the external standard method. At the three lowest levels, recovery tests were performed in fresh shiitake mushrooms, whereas at the 10 mg/Kg level, recovery tests were performed in dehydrated shiitake mushrooms. Different concentration levels were studied to verify the possibility of either assessing the compliance of the sample with the maximum residue level or ruling out the presence of residues. The value of the European maximum residue level established for benomyl in mushrooms is 1.0 mg/Kg (15,16). In Brazil, there are no maximum residue levels established for mushrooms. The average recovery results ranged from 76% to 86%, with relative standard deviation values of 1.1% to 5.8%, as shown in Table I. Each recovery analysis was repeated 6 times. The precision and accuracy were considered adequate for the validation of the method according to the validation criteria (17). The amounts of carbendazim obtained were corrected by the following factor to convert to the amounts of benomyl [mol. wt. of benomyl (290.4) / mol. wt. of carbendazim (191.2) = 1.51].

Table I. Recoveries of Benomyl from Fortified Shiitake Mushrooms Employing HPLC–UV

Shiitake	Spiked level (mg/Kg)	%Range of recovery* (%mean, %RSD†)
In natura	0.5	74–80 (78, 4.5)
	1.0	75–77 (76, 1.1)
Dehydrated	2.0	72–86 (80, 5.0)
	10.0	82–95 (86, 5.8)

* n = 6 replicates.
† RSD = relative standard deviation.

Table II. Benomyl Residues Detected in Shiitake Mushrooms after Benlate 500 Application

Shiitake	Treatment (µg/mL) (mg/Kg)	Range (mg/Kg)	Mean* (mg/Kg)
In natura	10 (2.0)	ND†	–
	50 (10.0)	1.7–2.2	1.9
	100 (20.0)	1.4–1.6	1.5
Dehydrated	10 (2.0)	1.1–2.3	1.7
	50 (10.0)	3.1–6.7	5.3
	100 (20.0)	4.8–9.2	6.3

* n = mean of duplicate analyses from 3 replicates.
† ND = not detected.

Under the chromatographic conditions described, the linearity of the assay was checked by calculating the regression line using the least squares method and expressed by the coefficient of determination, $r^2 > 0.998$. A seven-point calibration curve was obtained for carbendazim in the range of 0.4–50 µg/mL by plotting the recorded peak area versus the corresponding analyte concentrations.

The limit of quantitation (LOD) corresponded to the lowest fortification level assayed (0.5 mg/Kg) and a detection limit as low as 0.2 mg/Kg was estimated at the lowest concentration level assayed (18). The LOQ reached by a UV detector was good enough to determine residues at the maximum residue limit (MRL) set by the European Union.

Analysis of shiitake mushroom treated with Benlate 500

The aim of this preliminary study was to verify the possibility of shiitake mushroom (in natura and dehydrated) contamination by carbendazim residues, the degradation product of benomyl, due to the treatment with Benlate 500 to control opportunist fungi, such as *Poria sp* and *Trichodermas ssp*. The results of experiments showed that carbendazim residues were detected in all of the shiitake mushroom samples, except in the shiitake samples (in natura) treated at the dose of 10 µg/mL. Table II shows the results obtained from this experiment. A typical chromatogram of a shiitake sample obtained from experiments collected after Benlate 500 application (50 µg/mL) is given in Figure 5.

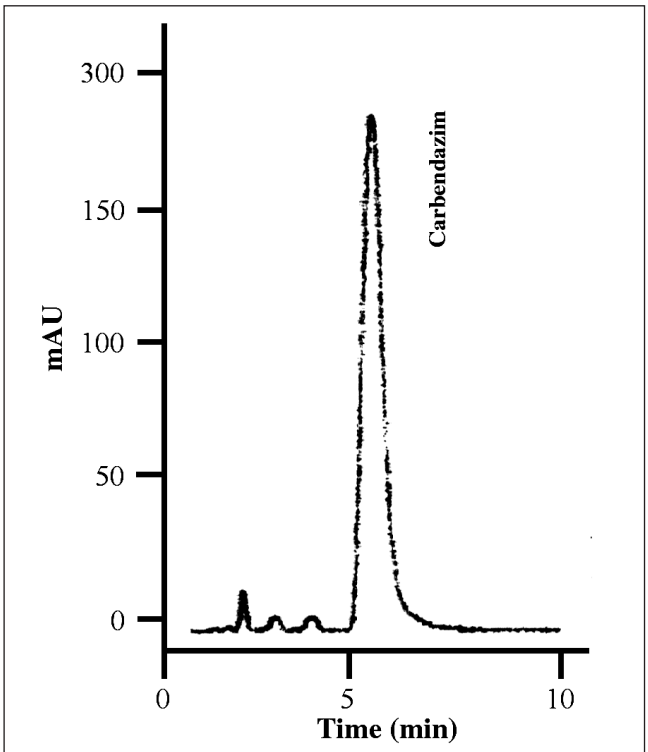

Figure 5. HPLC–UV chromatogram of shiitake in natura treated with Benlate 500 at 50 µg/mL. Retention time of the carbendazim peak is 5.4 min. For chromatographic conditions, see the Experimental section.

Conclusion

The proposed method allows a simple and rapid determination of carbendazim residues, the degradation product of benomyl, in shiitake mushroom. The method yields mean recoveries that range between 76–86%. The LOQ is lower than the MRL established by European legislation, which is indicative that the method used is valid for determination of residual levels of carbendazim derivative in this matrix. The application of the method to shiitake mushroom treated with Benlate 500 demonstrates that the concentration levels determined in the shiitake mushroom samples are higher than European MRL established for benomyl, except in the shiitake samples (in natura) treated at the dose of 10 µg/mL. These preliminary results suggest that if shiitake mushrooms are destined to be sold as a fresh or dried product, it may be advisable to lower the dose of the Benlate 500. Additional investigation should also be performed to better assess this fungicide behavior after scheduled treatments.

Acknowledgments

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