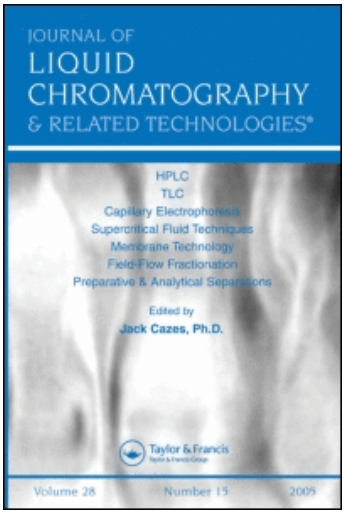

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HPLC WITH FLUORESCENCE DETECTION FOR THE STUDY OF BENOMYL DISSIPATION ON TREATED LETTUCES

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ABSTRACT

Benomyl is determined as carbendazim by using an analytical procedure that involves a previous lyophilization of the sample, then an extraction with ethyl acetate and after that a liquid-liquid partition. Carbendazim is evaluated by reversed-phase high performance liquid chromatography with fluorescence detection. The degradation rate of benomyl added on lettuces grown in greenhouses is strongly dependent on the temperature, which stablishes the security times for their trading.

INTRODUCTION

Benomyl and carbendazim are two related fungicides widely used on fruits and vegetables. Several authors have assigned to both fungicides a carcinogenic and mutagenic activity [1, 2], so their maximum residual levels allowed on vegetables are regulated in many countries in order to avoid potential toxic hazards. In order to know these levels and also the duration from the fungicide addition to that moment in which the residue concentrations are under the allowed limit, it is necessary to carry on studies about their persistence and degradation. There are several works about this theme, studying particular aspects such as the low penetration of the fungicides in the pulp of refrigerated fruits such as pears [3] and apples [4]. Their residual levels and accumulation in monarda and peppermint aromatic oils [5], strawberries [6], blueberries [7], and fruits as peaches and apples [8] have also been studied. Benomyl and carbendazim residues were also determined in liquid samples: wine, grape juice and water [9-11].

In most of the papers mentioned above, benomyl, due to its quick degradation rate, is usually

determined as carbendazim, introducing an acid hydrolisis step on the analytical procedure. Such procedure usually has an extraction step with organic solvents, one or more liquid-liquid partitions and then a HPLC determination with ultraviolet or fluorescent detection [6, 7, 9], but they have also been proposed other non-chromatographic techniques such as fluorescence, spectrophotometry, differential pulse polarography and inmunoassay among others [12-19].

In this work the dissipation of benomyl on treated lettuces growing in greenhouse has been studied, trying to relate such dissipation with the temperature and with the intensity of fungicide treatment. To this aim, an ordinary clean-up procedure with HPLC-fluorescence detection, adecuated for evaluating high levels of carbendazim, has been used; it has also been included a previous step of lyophilization of the sample [20]. The suitability of the analytical procedure has been tested on fortified samples of lettuce and tomato. Some considerations about the mobile phase composition, the wavelength selection and the stability of benomyl in aqueous solution have been made.

EXPERIMENTAL

Reagents

Residue analysis grade ethyl acetate, methanol, and *n*-hexane, and HPLC grade acetonitrile were purchased from Panreac (Barcelona, Spain) and SDS (Peypin, France). Ultrapure water was supplied by a Milli-Q plus apparatus from Millipore (Milford, Ma). Benomyl and carbendazim certified purity standards were supplied by Promochem (Wesel, Germany). Hydrochloric acid and sodium hydroxide were obtained from Panreac, and cellulose powder from Aldrich (Steinheim, Germany).

Apparatus

Fluorescence measurements were made on a Perkin-Elmer LS-5 computer-controlled spectrofluorimeter (Norwalk, Co).

Lyophilization equipment, furnished with a vacuum pump and a freezing system, was purchaded from Telstar (Barcelona, Spain) .

Turbo-vap evaporator system furnished with a thermostated water bath and nitrogen sweeping was

obtained from Zymark (Hopkinton, Ma). Kokusan centrifuge (Tokyo, Japan), universal food cutters and mechanic stirrers were also employed.

Sample preparation

Prior to extraction, an amount of 100 g of vegetable sample (lettuce leaves or tomato pulp and peel) was minced and blended with 20 g of powdered cellulose. Optionally, a known volume of a benomyl solution was added for spiking the sample. The mixture was then homogenized by shaking and frozen at -35°C for subsequent lyophilization.

Extraction

A lyophilized vegetable sample (0.2-2 g) was extracted by mechanical stirring with 10 ml of ethyl acetate for 10 min. The mixture was then centrifuged at 3000 rpm for 5 min and the organic phase was collected. The solid residue was extracted with another 10 ml of ethyl acetate, stirring for 10 min, and the liquid phase separated through centrifugation. The two organic phases collected were mixed and treated with 5 ml of 0.01 M HCl. After

stirring for 5 min, 5 ml of 0.1 M NaOH were added. The resulting phases were separated by centrifugation and the lower aqueous layer was discarded. The organic phase was evaporated to dryness under a nitrogen stream at 40°C. The extract was dissolved by shaking in 2 ml of methanol and 5 ml of *n*-hexane. After separation, the methanol extract was collected, filtered through a PTFE filter (0.5 μm pore size) and analysed by HPLC.

HPLC system

The HPLC system consisted of a ConstaMetric 4100 quaternary pump, an AutoMetric 4100 autosampler, a membrane degasser and a SpectroMonitor 3200 UV-visible detector, all of which was supplied by LDC Analytical (Riviera Beach, Fl), in addition to a Waters 470 fluorescence detector (Milford, Ma). Data were acquired and processed by means of a computerized system.

The chromatographic conditions used for the determination of carbendazim in the lettuce extracts were as follows: 150 x 4.6 mm Spherisorb ODS-2 column from Phenomex (Torrance, Ca), acetonitrile-water 30:70 as mobile phase at a flow-rate of 1.5 ml/min,

injected volume: 25 μl, excitation and emission wavelengths for fluorescence detection: 285 and 317 nm, respectively. Carbendazim from tomato samples was eluted with acetonitrile-water 20:80 at a flow-rate of 1.5 ml/min. Benomyl was determined using acetonitrile-water 50:50 as mobile phase at a flow-rate 1 ml/min and UV detection at 220 nm. The chromatographic column and the mobile phase were both thermostated at 25°C.

RESULTS AND DISCUSSION

Stability of benomyl in solution

A preliminary study about the stability of benomyl in aqueous solution was carried out by HPLC with UV detection in order to determine its degradation rate. The experiment was performed with the commercially available formulation Benlate, a preparation containing 50% benomyl, from Du Pont de Nemours (Wilmington, De) and a certified standard, all of which gave rise to a degradation profile such as that shown in Fig. 1. Benomyl peak area decreased by 50% within 7 h after preparation and by 70% after 15 h. The degradation rate was somewhat higher in the first hours.

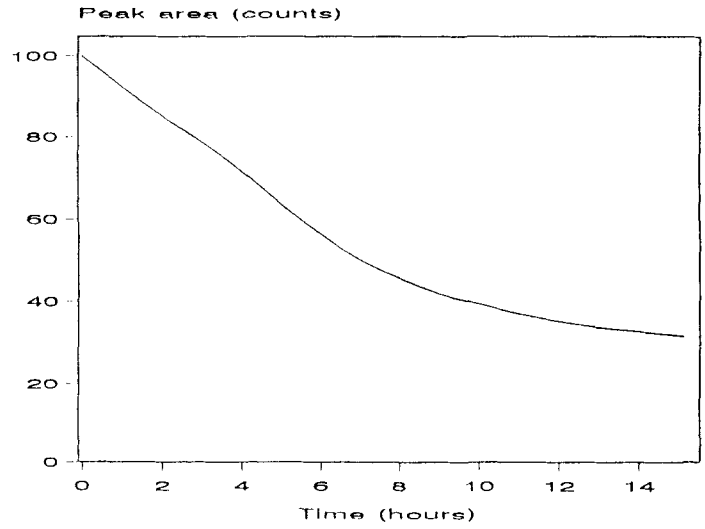


Figure 1: Degradation of benomyl in aqueous solution.

Since the degradation rate of benomyl is higher in organic solvents in relation to aqueous solutions [10, 21], laboratory determination is usually avoided. This led to evaluate the fungicide via its most common metabolite, carbendazim. The concentration of carbendazim analytically determined on vegetables will have two origins: the hydrolysis of benomyl and the carbendazim yielded by natural degradation under the prevailing environmental radiation, temperature and moisture conditions.

Selection of wavelengths

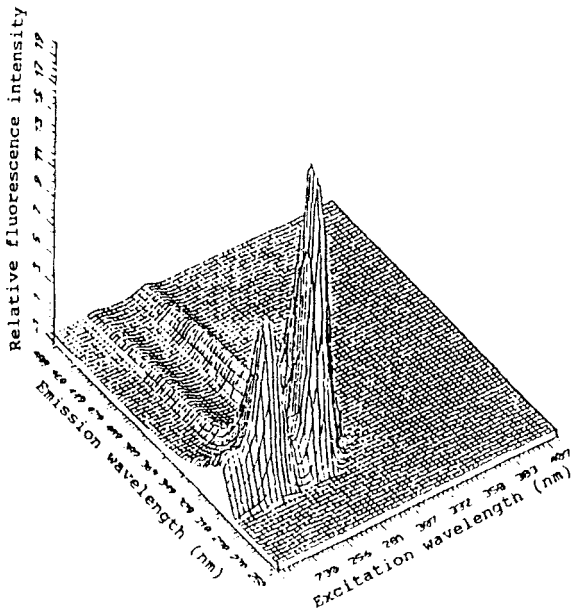
Figure 2 shows a three-dimensional representation, fluorescence intensity-excitation wavelength-emission wavelength, and its matching contour diagram for a methanol solution of carbendazim. The maximum fluorescence intensity was achieved at a excitation wavelength of 285 nm and a emission wavelength of 317 nm.

It is interesting to note that benomyl dissolved in methanol initially exhibits no fluorescence, only after a few hours a three-dimensional spectrum similar to that observed for carbendazim is obtained.

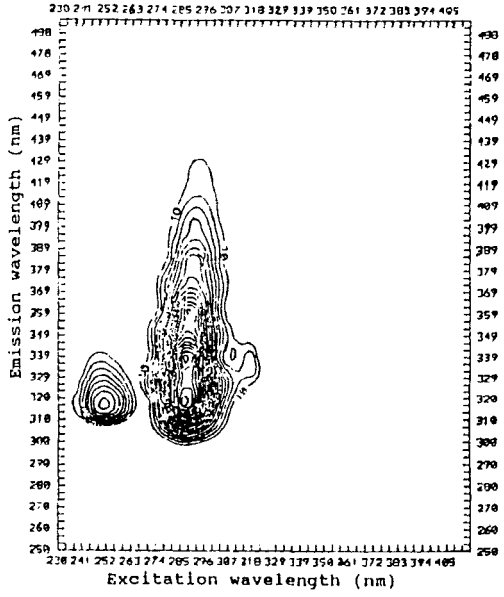
Analysis of fortified samples

Table 1 lists the results obtained by applying the extraction and clean-up procedure on fortified lettuce and tomato samples for proving the suitability of the method. The recoveries achieved for different matrices and fortification levels were all acceptable (above 95%), with a good relative standard deviation that ranged from to 3.3% to 3.8%.

The appropiate mobile phase varied as a function of the matrix due to the presence of different



Tridimensional fluorescence spectrum



contour map

Figure 2: Tridimensional fluorescence spectrum and contour map of a carbendazim standard dissolved in methanol.

TABLE 1

Recovery (%) of benomyl on fortified vegetable samples (n=5). Lyophilized sample amount: 2 g.

SAMPLE	FORTIFICATION LEVEL (mg/Kg)	RECOVERY (%)	σ_{n-1}
Tomato	6.0	95.3	3.4
Tomato	0.3	97.4	3.8
Lettuce	6.0	96.5	3.5
Lettuce	0.3	99.6	3.9

σ_{n-1}: standard deviation

coextracted substances. No such substances were found in the lettuce extracts, which mobile phase yielded a peak for carbendazim at a retention time of 2.8 min. On the other hand, tomato extracts contained unknown compounds whose chromatographic peaks overlapped with that of carbendazim under the conditions used for the lettuce extracts. The chromatographic peaks were isolated by using a different mobile phase, the elution of carbendazim was delayed to 5.7 min (Figure 3). Table 2 lists the retention times for carbendazim obtained with mobile phases of different composition.

The detection limit of carbendazim provided by the fluorescence detector was obtained by successive dilutions of a standard and found to be 0.07 mg/l. In consequence, the detection limit for the lettuce

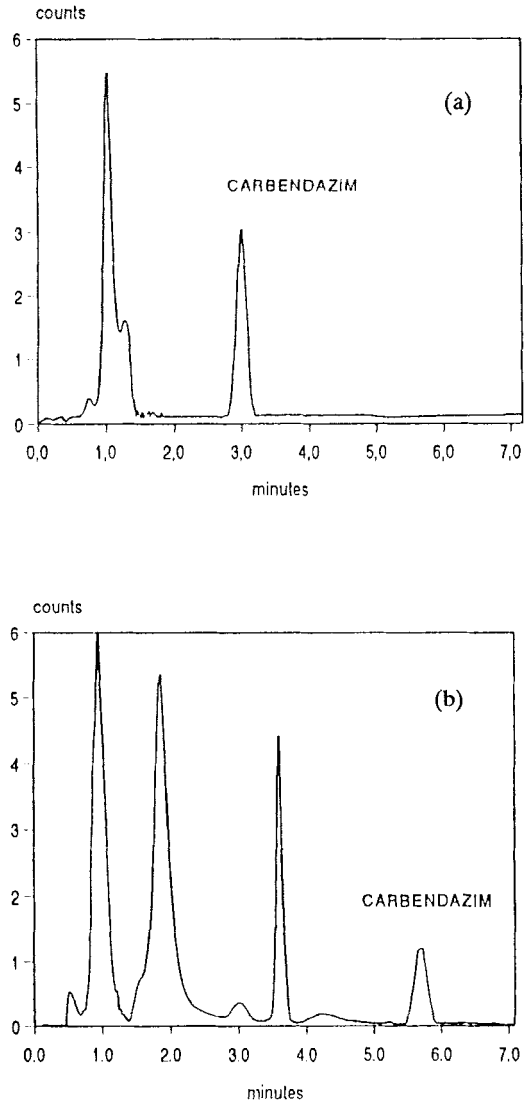


Figure 3: Chromatograms obtained by the proposed procedure.
3 a) Lettuce extract. Mobile phase: acetonitrile-water 30:70.
3 b) Tomato extract. Mobile phase: acetonitrile-water 20:80.

TABLE 2

Retention time (in minutes) of carbendazim on a 150 x 4.6 mm Spherisorb ODS-2 column. Flow-rate 1 ml/min.

ACN (%)	RETENTION TIME	
	ACN-WATER	ACN-BUFFER*
50	2.3	1.4
30	3.5	1.9
25	5.5	3.0
20	7.7	5.4

*Buffer: Na_2HPO_4/KH_2PO_4, 1/1 in volume, 0.03M each one.

samples was 0.03 mg/kg while the detection limit for the tomato analysis was somewhat higher, 0.05 mg/kg. Spanish legislation allows a maximum value of 5 mg/kg for leafy vegetables such as lettuces and 2 mg/kg for tomatoes, so the method complies with the legal limits for both products.

Dissipation of benomyl on lettuces

The above method has been applied to the determination of benomyl on greenhouse lettuce samples that were treated with a suspension (1 g/l) of Benlate, which was evenly spread on the vegetables.

Three randomly chosen individual lettuce heads were cut off at ground level, placed in plastic bags

and stored at -20°C in the dark, daily for 30 days.

The greenhouse was equipped with temperature sensors that allowed the average daily temperature to be known.

Figure 4 shows three dissipation curves of benomyl, in equivalent units of carbendazim, and the greenhouse temperature. As can be seen from one of the curves in the Fig. 4a, the initial concentration, 36.3 mg/kg, dropped off to 2.5 mg/kg in 30 days. The dissipation rate in the first week was quite low, in coincidence with prevailing low temperatures; later, the higher temperature seems to increase the dissipation rate. Finally, a virtually constant residual concentration is observed as a result of a slow dissipation rate observed although the high temperatures.

A similar behaviour was observed in the Fig. 4a, in the other experiment performed at similar temperatures and higher phytosanitary treatment intensity. The difference between the concentration levels of each experience decreased gradually, attaining a similar residual concentration, 2.5-3.0 mg/Kg, after 30 days.

As can be observed from Fig. 4b, the higher ambient temperature increased the dissipation rate.

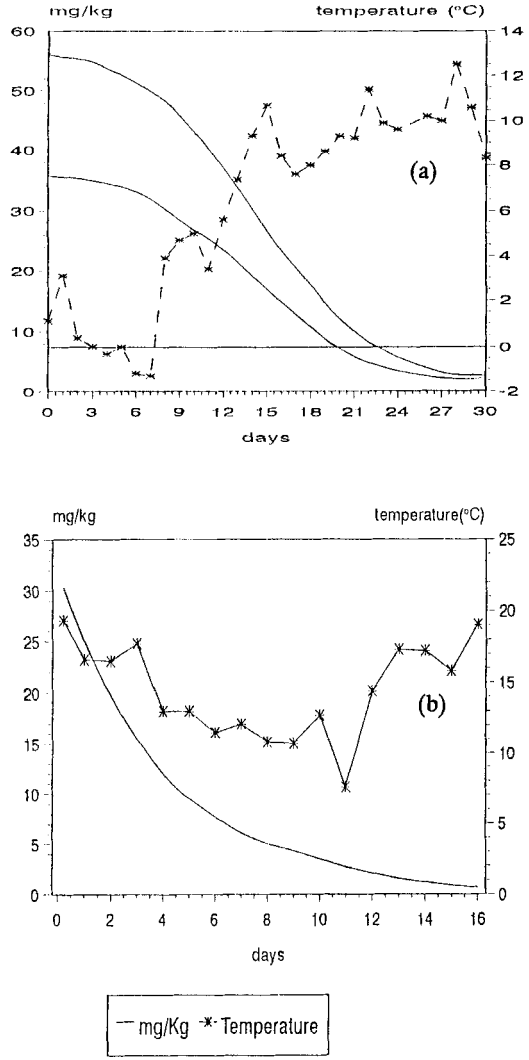


Figure 4: Dissipation of carbendazim on lettuces.
4 a) Low ambient temperature.
4 b) High ambient temperature.

In this case, the curve was not asymptotical in the first days after treatment; rather, the carbendazim concentration dropped off quickly from the beginning, from 30.3 to 0.7 mg/kg in 16 days.

The temperature can influence the dissipation rate in vegetables in two ways: through the dilution resulting from growth of the plant and through the rate of decay of the fungicide on the vegetable. Both are favoured by high temperatures.

On the basis of the maximum allowed carbendazim concentration (5 mg/kg), the vegetables studied would be fit for human consumption 22 and 24 days after treatment at low temperatures, and only 9 days after treatment at high temperatures. The interval of time for safe human consumption varies with the ambient temperature and the fungicide concentration used in the treatment.

CONCLUSIONS

The degradation rate of benomyl used on lettuces is strongly related to the environmental temperature, so higher is the temperature higher is the rate of degadation, which is very low at temperatures near 5 °C.

The degradation rate of benomyl is also affected by the intensity of fungicide treatment nevertheless its impact is lower than the temperature impact.

The use of lyophilizated sample simplifies the laboratory assays reducing the size of the necessary sample and the volume of organic solvents used.

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