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# Fluorimetric determination of thiabendazole residues in mushrooms using sequential injection analysis

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ABSTRACT

Thiabendazole is a benzimidazole fungicide of general use that is specifically used to control mushroom diseases, mainly cobweb diseases, which is caused by members of the genus *Cladobotryum*. Although this compound is legislated and its maximum residue limit established at 60 mg $kg^{-1}$ by Codex Alimentarius, there is almost a complete absence of analytical methods available for its determination in mushrooms. Here, we propose an automated method, using Sequential Injection Analysis with fluorescence detection ($\lambda_{exc}/\lambda_{em}$ = 305/345 nm) for the determination of thiabendazole in mushrooms. We have developed a flow-through optosensor using $C_{18}$ silica gel as solid support placed in the flow-cell where the determination is performed. This method presents a detection limit of 0.5 mg $kg^{-1}$, and recovery experiments have been carried out in different kinds of mushrooms at levels below the legislated maximum residue limit, demonstrating that the proposed analytical method fulfils the requirements for its applications in quality control of mushrooms.



## 1. Introduction

Modern agricultural production in major agriculture countries depends heavily on the use of phytosanitary products (insecticides, fungicides, herbicides) to control plagues and diseases in crops. As a result, there is an increasing unease related to the possible presence of residues from these products, which can present high toxicity, in the processed foods available to the consumer in the market. To ensure the safety of food for consumer, numerous legislations such as the European Union directives have established maximum residue limits (MRL) for pesticides in foodstuffs.

Thiabendazole is a benzimidazole fungicide that is widely used to control fruit and vegetable diseases such as mold, rot, blight and stain caused by various fungi. It is formulated as a ready-to-use, dusts, flowable concentrates, emulsifiable concentrates, wettable powders, granules, and water dispersable granules. This fungicide is registered for use on bananas, carrots, citrus fruits, mushrooms, pome fruits, potatoes, soybeans and wheat [1].

Thiabendazole, carbendazim, and prochloraz-Mn are the only fungicides that are typically recommended for controlling cobweb disease of mushrooms, which is caused by members of the genus *Cladobotryum*, including *C. dendroides* and *C. mycophilum* [2]. In a recent study, these three fungicides were tested for their efficacy in controlling cobweb disease caused by two *Cladobotryum* isolates. It was observed that one isolate was effectively controlled by thiabendazole and carbendazim and partially by prochloraz-Mn, while the other isolate was more resistant to the first ones [3]. In other research developed by the same author, the persistence of the fungicides in mushroom casing soil was studied [4], observing that the concentrations of carbendazim and prochloraz-Mn in the top half of the casing layer constantly decreased, whereas thiabendazole concentration was consistently high during the course of the crop. This is very important, as fungicides that do not persist at high concentrations in mushroom casing soil for the duration of the crop may not give good control of mushroom pathogens. From these studies, it can be concluded that each fungicide presents some handicaps when compared to the others. Finally, it is worth mentioning that these fungicides are also being tested nowadays for control of other diseases, such as web bubble disease, caused by mycoparasite *Mycogone perniciosa*, obtaining satisfactory results [5].

The maximum residue level (MRL) established for thiabendazole is 60 mg $kg^{-1}$ in mushrooms [6]. Human health risk assessment for this fungicide carried out by Environmental Protection Agency (EPA) indicates some risk concerns, specifically for children 1–6 years of age, with mushrooms driving the risk. As a result, EPA requires several risk mitigation measures, such as deletion of spray application to mushrooms, in order to lessen the risks of dietary exposure [1].

In general, the determination of pesticides residues in vegetables is a difficult task, not only because of the low concentration levels typically found, but also because of the complexity of

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the matrix, which usually involves various extraction and clean-up stages in sample treatment procedure. For this reason, highly selective and sensitive methodologies are required. In this sense, gas chromatography–mass spectrometry (GC–MS) or liquid chromatography–mass spectrometry (HPLC–MS) is usually selected for the analysis of a high number of pesticides in foods, including thiabendazole [7–14]. However, to the best of our knowledge, only one method has been developed for the determination of thiabendazole in mushrooms, using pressurized hot water in the extraction process and determining the analyte by HPLC with UV absorbance and fluorescence detection [15].

In this paper, we propose a rapid and simple method for the determination of thiabendazole in mushrooms. The extraction procedure used has been the well-known QuEChERS method [16,17]. We have made use of the coupling of solid-phase spectroscopy (SPS) and flow analysis to develop the analytical method. This methodology, called flow-through optosensing, consists in using a solid support to retain and preconcentrate the analyte in the detection area, followed by non-destructive spectroscopic measurements (in this particular case, fluorescence) on the same solid-phase, being all the steps carried out on-line [18–21]. In this way, high sensitivity and selectivity are obtained. The methodology used in this system has been Sequential Injection Analysis (SIA), which provides the method with the required automation. The proposed method has been applied to different kinds of mushrooms, performing recovery experiments to validate it, obtaining recovery values close to 100% in all cases. In addition, an HPLC reference method has been used.

## 2. Experimental

### 2.1. Reagents and solutions

Thiabendazole (Riedel-de-Haën) stock solution of 200 μg ml$^{-1}$ was prepared by weighing the appropriate amount of the compound and dissolving it in methanol. The solution was kept away from light at about 4 °C.

Methanol (analytical reagent grade, used in the proposed method), acetonitrile, sodium chloride and magnesium sulphate anhydrous were obtained from Panreac. $C_{18}$ bonded phase silica gel beads (Waters, Milford, USA) with 55–105 μm of average particle size, was used as solid support. Supelclean PSA SPE (Supelco) (primary secondary amine, solid phase extraction) bulk packing was used in the clean-up stage of the extraction procedure.

Methanol (HPLC grade, Panreac) was used for chromatographic separation. Methanol–water (50:50, v/v) was used as isocratic mobile phase at a flow rate of 1 ml min$^{-1}$.

### 2.2. Apparatus and instruments

A FIALab-3500 analyzer (FIALab Instruments, Inc., the USA) was used in the manifold. The analyser includes two 5-ml syringe pumps (flow-rate from 2 to 250 μl s$^{-1}$), a holding coil, a peristaltic pump and a 10-port multiposition valve, being all the components computer-controlled by FIALab 5.0 software. The analyser was connected to a Cary-Eclipse Luminescence Spectrometer (Varian Inc., Mulgrave, Australia) for luminescence measurements. A Hellma flow cell 176.752-QS (25 μl of inner volume and a light path length of 1.5 mm) was used. The cell was filled with $C_{18}$ solid phase microbeads, and was blocked at the outlet with glass wool to prevent displacement of the solid support.

The HPLC apparatus comprised a Shimadzu SCL-10A vp (USA Manufacturing) controller with a Rheodyne model 7725 injection valve equipped with a 20 μl loop. A Shimadzu SPD-10AV vp pump was used and all data (fluorescence detection, $\lambda_{exc}/\lambda_{em}$ = 305/345 nm) were recorded by using LC Solutions software. A Kromasil-100-$C_{18}$ column (150 mm × 4 mm i.d., 5 μm particle diameter) from Tecknokroma (Barcelona, Spain) was used and thermostated at 55 °C. Methanol–water (50:50, v/v) was used as isocratic mobile phase at a flow rate of 1 ml min$^{-1}$. The method was adapted from a previously reported method [22].

### 2.3. Sample treatment

The QuEChERS method, which involves an acetonitrile extraction/partitioning and dispersive solid-phase extraction (SPE) clean-up with primary secondary amine (PSA) was used to extract thiabendazole from mushrooms [17]. The dispersive-SPE with PSA removes many polar matrix components from the food extracts, such as organic acids, pigments or sugars, removing potential interference compounds.

Samples were obtained from local markets. The matrix-matched standards were prepared from sample extracts that were checked by the HPLC reference method in order to ensure the absence of thiabendazole. 500–1000 g of mushroom were chopped and homogenized with a high-speed laboratory homogenizer. A representative 10-g portion of this sample was weighed in a 50 ml PTFE centrifuge tube and 10 ml of acetonitrile was added. The screw cap was closed and the tube was vigorously shaken for 1 min by hand ensuring that the solvent interacted well with the entire sample. Then, 2 g of NaCl and 4 g of $MgSO_4$ anhydrous were added, repeating the shaking process for 1 min to prevent coagulation of $MgSO_4$. After this time, it was centrifuged at 3700 rpm for 4 min. 4 ml of the supernatant (acetonitrile phase) was taken with a pipette and transferred to a 15 ml centrifuge tube. After adding 200 mg of PSA and 600 mg of $MgSO_4$, the tube was energetically shaken for 20 s by hand and centrifuged again (3700 rpm) for 3 min. Finally, an extract containing 1 g of sample per ml in 100% acetonitrile was obtained. This extract was further diluted with deionized water prior to analysis by the proposed method.

### 2.4. General procedure

Before starting the analysis of any sample, 30% (v/v) MeOH carrier solution (3000 μl) was first aspirated into the syringe pump, at a flow rate of 10 ml min$^{-1}$, from a reservoir, and passed through the sensing zone in order to condition it. This solution will be used as carrier for delivering both sample and eluting solutions towards the flow cell.

Once the solid support is conditioned, the procedure used for the analysis of each sample is as follows: (a) 400 μl of carrier and 200 μl of sample were sequentially aspirated into the syringe pump and holding coil respectively at a flow rate of 10 ml min$^{-1}$ and pumped towards the waste in order to avoid contamination with the previous sample; (b) 800 μl of carrier and 500 μl of sample were aspirated at 10 ml min$^{-1}$ and pumped towards the flow-cell at 1.2 ml min$^{-1}$, recording the analytical signal from thiabendazole; (c) 400 μl of MeOH were aspirated and pumped through the solid support at 1.2 ml min$^{-1}$ in order to completely regenerate it. Hence the system was prepared for a new sample injection.

The instrumentation is shown in Fig. 1, where only the components of the FIALab that have been used in this method are represented, for simplicity's sake.

## 3. Results and discussion

### 3.1. Preliminary studies

As it has been already reported [23], $C_{18}$ bonded silica gel beads provide the highest enhancement in the analytical signal when the retention of thiabendazole is performed on different solid supports. Hence, $C_{18}$ silica gel has been chosen for the development of this

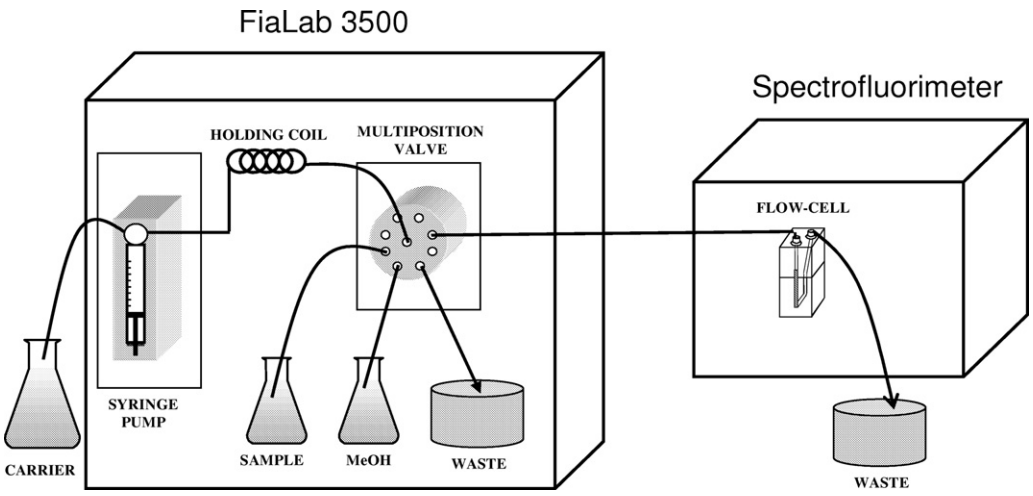


**Fig. 1.** Instrumentation used in the proposed method.

analytical method. More than 100 sample determinations could be performed without replacing the solid support in the flow-cell.

The spectral features of thiabendazole were recorded with the analyte retained on the $C_{18}$ solid support in the flow-cell. Fluorescence spectra, which were collected in working aqueous solution, showed maxima excitation/emission wavelengths at 305/358 nm, which were the selected excitation and emission wavelengths to carry out the determination of thiabendazole in the proposed method.

Relative fluorescence intensity measurements carried out in gel-phase media are usually affected by background signal levels higher than those found in homogeneous solution, obviously owing to the presence of the solid sensing support in the irradiated zone. Therefore, instrumental parameters and conditions of measurement have to be carefully investigated in order to achieve the best possible signal-to-background ratio. For this reason, the influence of the voltage of the photomultiplier tube (400–800 V) and the instrument excitation and emission slit widths (5–20 nm) on the analytical signal had to be studied. As a compromise between sensitivity and signal-to-background ratio, the photomultiplier tube voltage was set at 650 V and the excitation and emission slit widths were fixed at 5 and 10 nm, respectively.

### 3.2. Chemical variables

The nature of carrier and eluting solutions were the chemical variables under investigation. The extract of the sample was diluted 50-fold with deionized water prior to analysis, in order to avoid interferences from the matrix.

Taking into account the non-polar nature of $C_{18}$ silica gel beads, several carrier solutions were tested containing methanol and water in different proportions, ranging from 10 to 40% MeOH (v/v). When the percentage of methanol increases, the elution of the analyte is faster, but the analytical signal diminishes due to a lower retention and preconcentration of the analyte on the solid beads. The best overall results were obtained with 30% MeOH (v/v) solution, obtaining satisfactory sensitivity and complete desorption of thiabendazole from the support.

In addition, although the baseline could be completely regenerated by the carrier solution, it was observed that this regeneration was slow and that, after several sample insertions, the repeatability of the system worsened (probably due to the retention of some components of the matrix on the solid beads). In order to avoid these handicaps, 400 μl of MeOH was inserted after each sample determination, improving the repeatability and sample throughput of the system.

### 3.3. SIA variables

The studied SIA variables were the volumes of solutions and the effect of the flow-rate. The sample volume was studied ranging from 100 up to 1200 μl. When increasing the volume of the sample aspirated, the amount of analyte sorbed on the beads also increases; hence, the analytical response and sensitivity are higher, but the sampling frequency diminishes. In this case, 500 μl of sample solution was the chosen volume, as enough sensitivity was obtained for the required applications, achieving good sampling frequency. In the case of using MeOH as eluting solution, 400 μl was observed to completely regenerate the sensing support.

For the aspiration of solutions, 10 ml $min^{-1}$ flow-rate was selected in all cases, in order to shorten the time of analysis. For the process of delivering solutions towards the flow-though cell, the flow-rate was investigated from 0.5 to 2 ml $min^{-1}$ (by inserting a sample solution corresponding to 30 mg $kg^{-1}$ thiabendazole). By increasing it, the sampling frequency increased but the analytical signal decreased (due to a lower retention of the analyte on the solid beads) and over-pressure could appear in the system. The signal was observed to decrease very slightly up to 1.2 ml $min^{-1}$. Therefore this last one was the chosen flow-rate as a compromise between sensitivity and sample-throughput.

### 3.4. Analytical parameters

The analytical parameters of the system were studied using the optimized conditions previously discussed. Table 1 contains the figures of merit of the proposed method using a sample volume

**Table 1**
Analytical parameters.

| Parameter | |
|---|---|
| Linear dynamic range/mg $kg^{-1}$ | 1.6–40 |
| Calibration graph | |
| Intercept | 1.5 |
| Slope/kg $mg^{-1}$ | 19.65 |
| Correlation coefficient | 0.9992 |
| Detection limit/mg $kg^{-1}$ | 0.5 |
| Quantification limit/mg $kg^{-1}$ | 1.6 |
| Intra-day RSD (%) ($n$ = 10) | 2.6[a] |
| Inter-day RSD (%) ($n$ = 10) | 5.7[a] |

[a] For a concentration level of 20 mg $kg^{-1}$.

of 500 μl. The calibration curve was constructed using matrix-matched standards (sample extracts fortified with thiabendazole at different levels) due to the matrix effect observed in the samples. The data were fitted by standard least-squares treatment and the calibration equation is shown. The proposed methodology was able to produce analytical fits with good linearity in the range 1.6–40 mg $kg^{-1}$. Inter- and intra-day repeatabilities were established for ten independent analyses of sample solutions containing 20 mg $kg^{-1}$ of thiabendazole. The limit of detection was estimated from the injection of matrix-matched standard solutions, and corresponds to a signal-to-noise ratio of three. The method meets the requirement of MRL established by Codex Alimentarius [6].

### 3.5. Selectivity study

In order to check the usefulness of the proposed method for analysing real samples, foreign species that are likely to be present in real samples were added to solutions containing 10 mg $kg^{-1}$ thiabendazole, and their influence on the analytical signal was investigated. Tolerance level was defined as the amount of foreign species that produced an error not exceeding $\pm 2\sigma$ in the determination of the analyte, being $\sigma$ the standard deviation. The study of potential interferences was carried out with other pesticides that are widely used, even in commercial pesticide formulations along with thiabendazole, such as imazalil.

The maximum tolerated ratio (interference/analyte, w:w) was higher than 100 for aminocarb, bendiocarb, benomyl, chlorsulfuron, imazalil, imazaquin, morestan, quinmerac and simazine, 15 for α-naphtol, carbaryl, carbendazim, and 5 for o-phenylphenol. It can be observed that thiabendazole can be analysed, without significant errors, in the presence of levels of potentially interfering compounds higher than those likely to be present in real samples.

### 3.6. Recovery study

The chosen mushroom samples were selected in different presentations: fresh or tinned and whole or laminated. In all cases, the samples were analysed by both the proposed method and the reference method [22]. It was observed that thiabendazole levels were below the detection limit of the proposed method. Therefore, a recovery study was carried out to evaluate the accuracy of the whole method (extraction and determination). The recovery experiments were accomplished by spiking mushroom samples at different concentration levels, being then extracted and analysed with the developed method.

A representative 50-g portion of a blank mushroom sample previously homogenized was weighted and transferred to a glass mortar, where it was fortified with the required volume of the 200 mg $ml^{-1}$ thiabendazole standard solution (prepared in MeOH). The mixture was then gently blended in the mortar to assess the homogeneity of the sample. The sample was allowed to stand at room temperature for 12 h and kept in the fridge until the extraction method and analysis were performed. The recoveries, shown in Table 2, ranged between 93 and 110% in different species and presentations of mushrooms. The results obtained by the HPLC reference method have also been included in this table.

Statistical analyses of the results obtained were performed with two objectives: (a) to evaluate the accuracy of the recovery experiments in the proposed method; (b) to compare if there was significant difference between the proposed method and the reference one with regard to accuracy.

(a) The method of the average recovery was performed as a significant test in order to assess the accuracy of the method [24]. The average recovery is tested for significance by using Student's $t$ test, the null hypothesis being that the recovery is the unity.

**Table 2**
Recovery studies of thiabendazole in mushrooms.

| Sample | Spiked (mg $kg^{-1}$) | Recovery (%)[a] | | $t_{exp}$ |
|---|---|---|---|---|
| | | Proposed method | Reference method | |
| *Agaricus bisporus* (fresh, laminated) | 20 | 96 (3) | 98 (4) | 0.69 |
| | 40 | 94 (4) | 97 (1) | 1.26 |
| | 60 | 99 (3) | 102 (3) | 1.22 |
| *Pleurotus ostreatus* (fresh, whole) | 15 | 106 (5) | 102 (3) | 1.19 |
| | 30 | 97 (3) | 105 (5) | 2.38 |
| | 40 | 105 (4) | 99 (3) | 2.08 |
| *Agaricus bisporus* (tin, whole) | 20 | 93 (3) | 95 (2) | 0.96 |
| | 40 | 98 (3) | 96 (4) | 0.69 |
| | 60 | 102 (4) | 98 (3) | 1.38 |
| *Lentinus edodes* (fresh, whole) | 10 | 110 (5) | 105 (6) | 1.11 |
| | 20 | 104 (5) | 99 (4) | 1.35 |
| | 50 | 95 (3) | 99 (4) | 1.38 |
| *Lactarius deliciosus* (tin, whole) | 25 | 94 (3) | 97 (2) | 1.44 |
| | 50 | 109 (4) | 107 (2) | 0.78 |
| | 75 | 97 (2) | 101 (4) | 1.55 |
| *Pholiota aegerita* (fresh, whole) | 40 | 106 (3) | 107 (4) | 0.35 |
| | 60 | 105 (4) | 108 (4) | 0.92 |
| | 80 | 98 (3) | 105 (5) | 2.08 |

[a] RSD (%) in parenthesis; $n = 3$.

The observed $t$ value, being Rec the mean recovery, is calculated as:

$$t = \frac{\text{Rec} - 1}{s_{\text{Rec}}/\sqrt{n}}$$

This value is compared with the tabulated value for $n-1$ degrees of freedom. If the experimental $t$ value is less than the tabulated one, the null hypothesis is accepted and the method is accurate. The obtained $t$ value in the proposed method ($t = 0.335$, $n = 18$) was lower than the tabulated one ($t = 2.110$, 95% confidence level), therefore demonstrating the accuracy of the SIA method.

(b) The comparison between the accuracy of the SIA and reference methods was also performed to test if there was any significant difference between them [25].

For the comparison of the average values obtained with both methods, the standard deviation of the system, $\sigma$, and the experimental $t$, $t_{exp}$, were calculated.

The theoretical value for $t$ at a confidence level $P = 0.05$, $n = 3$, is 4.30. The $t_{exp}$ values obtained for each spiked sample were lower than 4.30 in all cases, showing that there was no significant difference between the proposed method and the reference one. These values are shown in Table 2.

### 3.7. Ruggedness and robustness

This study was performed for the determination of 30 mg $kg^{-1}$ thiabendazole in fresh, laminated *Agaricus bisporus*. The ruggedness of the method was assessed by comparison of the intra- and inter-day assay results undertaken by two analysts. The RSD values (%) for intra- and inter-day assays did not exceed 6 and 9%, respectively, indicating the ruggedness of the method.

The robustness of the analytical method, which can be described as its capacity to remain unchanged when there are small variations in experimental conditions, was also investigated. In this case, small changes were carried out in the carrier solution (25–35% MeOH (v:v)) and in the flow-rate (1–1.4 ml $min^{-1}$). The recoveries were in the 92–107% range in all cases, therefore demonstrating the robustness of the proposed method.

## 4. Conclusions

In this work, we have evaluated the potential of using SIA-optosensing for the analysis of residues of thiabendazole in mushrooms. The proposed method, which makes use of the QuEChERS method for the extraction and sequential injection analysis for its determination, is simple, rapid, sensitive, selective and cost-effective. The extraction procedure and the solid support ($C_{18}$ silica gel) used in the flow-cell allow the sensitive and selective determination of the target compound without extensive and laborious pre-treatment. The obtained analytical features meet the regulation established for thiabendazole residues in mushrooms (MRL of 60 mg $kg^{-1}$). Statistical treatments have been carried out to check the accuracy of the proposed method and compare it to the HPLC reference method. The obtained data support the usefulness of the SIA methods as a suitable alternative to chromatographic techniques and it can be easily used in routine analysis for the control of fungicide residues in mushrooms.

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