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Screening method to detect difenoconazole and cyproconazole in soils

Ana Paula Ferreira de Souza
University of Campinas, Brazil
E-mail: anapfs@unicamp.br, Orcid: https://orcid.org/0000-0002-7870-4409

Nadia Regina Rodrigues
University of Campinas, Brazil
E-mail: nadia@cpqba.unicamp.br, Orcid: https://orcid.org/0000-0002-5524-9781

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Abstract

Cyproconazole (CYP) and difenoconazole (DIF) are triazole fungicides widely used in agricultural areas. They are applied to protect plants against fungal pathogens; however, they negatively impact soil microbial diversity, particularly bacterial populations. This highlights the need for residue monitoring. In this study, a method was developed and validated to easily determine these residues in soils. The soil samples were extracted using a methanol:water solution and injected directly into a liquid chromatograph with an ultraviolet detector. The method demonstrated a limit of quantification of 0.2 mg kg-1 for both CYP and DIF, with an associated uncertainty of 11.5%. Residues of these compounds were not quantified in the soil samples tested. This technique is cheap, easy to execute, and can be applied as a screening method.

Keywords: chromatography; contamination; pesticides; fungicides.

Resumo

Método de triagem para detectar difenoconazol e ciproconazol em solos

Ciproconazol (CIP) e difenoconazol (DIF) são fungicidas triazólicos amplamente utilizados em áreas agrícolas. Aplicados para proteger plantas contra patógenos fúngicos pela inibição do ergosterol e com impacto negativo na diversidade microbiana do solo, especialmente nas populações bacterianas, destacando a necessidade do monitoramento de resíduos. Neste estudo, desenvolveu-se e validou-se um método simples para determinar esses resíduos em solos. As amostras de solo foram extraídas com uma solução metanol: água e injetadas diretamente no cromatógrafo líquido, com detector ultravioleta. O método apresentou limite de quantificação de 0,2 mg kg^{-1} tanto para CIP quanto para DIF, e a incerteza de medição associada atingiu 11,5%. Os resíduos desses compostos não foram quantificados nas amostras de solo testadas. A técnica analítica empregada é barata, de fácil execução e pode ser aplicada como método de triagem.

Palavras-chave: cromatografia; contaminação; agrotóxicos; fungicidas.

Resumen

Método de selección para detectar difenoconazol y ciproconazol en suelos

Ciproconazol (CIP) y difenoconazol (DIF) son fungicidas triazólicos ampliamente utilizados en el ámbito agrícola. Se aplican para proteger las plantas contra patógenos fúngicos mediante la inhibición del ergosterol y tienen un impacto negativo en la diversidad microbiana del suelo, especialmente en las poblaciones bacterianas, lo que destaca la necesidad de monitorear los residuos. En este estudio se desarrolló y validó un método sencillo para determinar estos residuos en suelos. Las muestras de suelo se extrajeron con una solución metanol:agua y se inyectaron directamente en el cromatógrafo líquido, con un detector ultravioleta.

El método presentó un límite de cuantificación de 0,2 mg kg^{-1} tanto para CIP como para DIF, y la incertidumbre de medición asociada alcanzó un máximo de 11,5%. No se cuantificaron residuos de estos compuestos en las muestras de suelo analizadas. La técnica analítica empleada es económica, de fácil ejecución y puede aplicarse como método de selección.

Palabras clave: cromatografía; contaminación; agrotóxicos; fungicidas.

Introduction

Brazil is among the world's major agricultural producers. In 2024, the country had approximately 94.8 million hectares of cultivated land, predominantly used for rice, corn, soybeans, wheat, cotton, and coffee (IBGE, 2025). Agriculture has expanded significantly in recent decades, and as a result, the use of pesticides has increased. In 2020, approximately 4.3 kg ha^{-1} of pesticides were used in planted areas, potentially affecting the environment and soil biota (MAPA, 2019). Among the active ingredients sold, 55.96% are herbicides, 17.71% are fungicides, and 12.14% are insecticides (IBAMA, 2025).

Triazolic fungicides have been produced since 1973. They share a common 1,2,4-triazole ring structure. These fungicides exhibit a broad spectrum of action against diseases caused by Ascomycetes, Basidiomycetes, and Deuteromycetes in different crops, including cereals, soybeans, rice, grapes, potatoes, and several vegetables (WHO, 2020). They are registered for use with foliar application and soils in Brazil (ANVISA, 2022). Additionally, these fungicides act directly on the cytoplasmic membrane of fungi by inhibiting the activity of the enzyme 14R-demethylase, which is involved in ergosterol biosynthesis (Sant *et al*., 2016; Thabit *et al.*, 2021).

These compounds are frequently detected in agricultural soils, primarily because of their physicochemical properties, such as high octanol–water partition coefficients and a high degree of persistence in soil (Granados *et al*., 2025). Variations in the adsorption and absorption indices in soils with different properties influence the leaching and dispersion of these fungicides in the environment (Pinheiro; Moraes; Silva, 2011; Felicio *et al*., 2016; Bošković *et al*., 2020; Ramborger *et al*., 2020; Scorza Júnior *et al*., 2021).

Capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC), which is often paired with ultraviolet (UV) or mass spectrometry (MS) detection, are methods widely used to determine fungicide residues (Miyauchi *et al.,* 2005; Gerónimo *et al*., 2015; Felicio *et al*., 2016; Takala *et al*., 2019; Scorza Júnior *et al*., 2021). Sample preparation is often a long process that consumes large amounts of solvent. To address these challenges, several authors have optimized extraction techniques to promote green chemistry, generating less chemical waste and minimizing environmental impacts (Santos *et al*., 2021; Câmara *et al*., 2022).

Thus, Cyproconazole (CYP) and Difenoconazole (DIF) were chosen as representatives of the triazolic class, and this work reports a method based on liquid chromatography with ultraviolet-visible detector (UV–Vis). The method was validated and applied to real soil samples, making it a suitable screening tool for soil analysis.

Material and Methods

Chemicals and reagents

Standards of CYP and DIF were purchased from Sigma-Aldrich, with a purity of greater than 97%. The stock standard solutions were individually prepared in acetonitrile and stored at -20 °C.

HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from JT Baker (USA). Ammonium acetate (NH_4Ac) (99.9%) was added to the mobile phase from JT Baker (USA). Water was obtained from a Milli-Q water system (Millipore, Bradford, MA, USA).

Soil sample preparation

The soil samples were collected exclusively from the 0 to 10 cm surface layer of a vegetable farm in the Campinas region, São Paulo, Brazil (predominantly Ultisol soil). After removing grass and other debris, the

soil samples were dried in an oven for 24 hours at 40 °C and then sieved through a 2 mm mesh. They were homogenized and stored at -20 °C until analysis. Soil moisture content was determined by drying a portion of the samples in an oven at 105°C to a constant weight.

The soil sample (10.0 g) was weighed into a 50 mL centrifuge tube, and 8 mL of the extraction solution (MeOH: H2O, 80:20 v/v) was added. Then, the sample was shaken in a shaker at 300 rpm for 20 min and centrifuged at 5000 rpm for 5 min. The supernatant was filtered through a glass filter using Whatman # 4 paper, and the filtrate was collected in a graduated cylinder. The procedure was repeated, and the supernatants were grouped (approximately 12 mL). Then, an aliquot of these supernatants was filtered through Millex 0.45 μm filters into 1.8 mL vials.

HPLC-UV analysis

The analyses were performed on an HPLC Shimadzu LC-20AD (Japan) equipped with an ultraviolet detector model SPD-10AVP. The compounds were separated under isocratic conditions using a C18 column (Microsorb Symmetry, 150 mm x 3.9 mm, 5 μm) at 50 °C. The mobile phase was ACN: NH_4Ac at 1 mmol L^{-1} (60:40 v/v) with a flow rate of 1.0 mL min^{-1} and a wavelength of 220 nm. The analytical column was a Microsorb Symmetry C18 (150 mm x 3.9 mm 5 μm), and the injection volume was 50 μL. Data acquisition was performed by LC Solutions (Shimadzu, Japan).

Quality assurance and quality control

Method validation and performance to quantify the residues were conducted in accordance with quality control standards following the European Commission (EC) guidance document for pesticide residue analysis in food and feed (Sanco, 2014). The considered parameters were selectivity, linearity, precision, recovery, and the limit of quantification (LOQ) and detection (LOD). In addition, storage stability was evaluated, and uncertainty measurement was performed using the top-down approach according to the EURACHEM/CITAC Guide (EURACHEM, 2000).

Selectivity was observed by comparing the blank sample with the spiked sample using CYP and DIF standard solutions. Linearity was assessed at concentrations of 0.1, 0.2, 0.4, 0.8, and 1.0 μg/mL for both pesticides, with five replicates. The least-squares method was used, and a correlation coefficient (R) above 0.99 was considered acceptable. The Huber test was applied to identify outliers, and the Cochran test was used to confirm homoscedasticity. Additionally, residual analysis was performed based on a normal distribution of the points on the analytical curve, following the deviation criteria (±20%).

The LOQs were determined through spiked sample analysis in the lowest concentration with acceptable precision (CV ≤ 20%) and recovery (70–120%). The LOD was determined as three times the signal-to-noise ratio. Precision and accuracy were evaluated through fortification assays with blank soil at two levels for each pesticide. The lowest level was the LOQ, and the highest level was three times the LOQ. Two analysts conducted precision and accuracy tests on different days. Accuracy was assessed by expressing recovery as a percentage (%), while method precision was measured using the coefficient of variation (CV%).

The storage stability of CYP and DIF was measured in standard solutions stored under two different conditions, at room temperature for 24 hours and in a refrigerator (5 ± 2 °C), for 75 days. All statistical analyses were performed using *Statistica* 7.0 software (StatSoft Inc., Oklahoma, USA).

Discussion

Chromatographic separation and sample preparation

The use of triazolic fungicides, such as CYP and DIF, in crops can lead to the accumulation of these pesticides in environmental compartments, such as soil and water. The persistence of triazole fungicides in the soil is a significant environmental concern, and dissipation studies, as described by Granados *et al.* (2025), have assessed the risk of long-term contamination. The persistence of DIF is considered high in the soil, with a half-life exceeding 115 days in field trials. They also reported that the persistence of DIF is greater in clay soils and

when it is applied at higher doses, potentially lasting up to 251 days (Granados *et al*., 2025).

In addition, owing to their toxicological characteristics and effects on humans and animals, several triazolic compounds, including CYP and DIF, have been classified by the WHO as moderate-risk substances (class II) (WHO, 2020).

Soil is a complex and heterogeneous entity, and its properties are influenced by elements such as soil microbiota, nutrients, and soil organic matter (Ramborger *et al*., 2020; Jakl *et al*., 2021). Given the complexity of the matrix, efficient sample preparation with a cleanup step is necessary to eliminate potential interference.

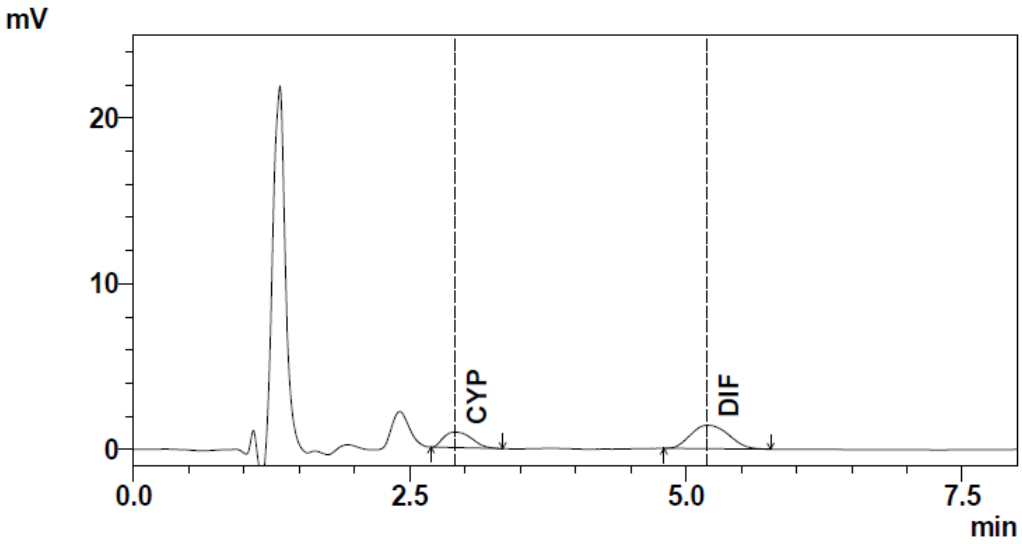
Granados *et al.* (2025) employed the QuEChERS method, using 5 g of soil, followed by a cleanup step with C18, which proved to be a practical and robust approach for DIF extraction. On the other hand, Miyauchi *et al.* (2005), although working with a different matrix (wood), used solid-phase extraction (SPE) with Oasis MCX for the determination of CYP and tebuconazole (TEB).

The method proposed by Granados *et al.* (2025) is ideal for detailed, fundamental research studies, as it requires advanced equipment, making it less practical for large-scale, routine monitoring. The authors employed a cutting-edge analytical technique (UHPLC-Q Exactive Orbitrap−MS), which enables not only the precise quantification of pesticides with high sensitivity and selectivity but also the identification of their metabolites. Miyauchi *et al.* (2005) analysed CYP using a detection method similar to that presented in this study (LC-UV).

The partition coefficients (log P) of the studied compounds were 4.3 for DIF and 3.1 for CYP. These compounds are highly soluble in organic solvents such as dichloromethane and acetone, as well as in more environmentally friendly solvents such as methanol. Therefore, the extraction method used in our study eliminated this cleaning step, which lowered costs, reduced the number of sources of experimental errors, and minimized environmental impact. Performing two extractions with an 80:20 (v/v) MeOH:H2O mixture proved to be the best option for improving the recovery of CYP and DIF.

Therefore, to achieve suitable simultaneous analyses of these fungicides in terms of selectivity and peak shape, different chromatographic parameters were tested. Organic solvents (ACN and MeOH) and the modifier NH_4Ac were tested, and the composition and proportion of the mobile phase that provided better resolution was 60:40 (v/v) of ACN: NH_4Ac 1 mmol L^{-1}. The separation column, combined with the composition and flow rate of the mobile phase, provided good chromatographic separation, allowing a total run time of 15 minutes. The retention times to CYP and DIF were 2.9 min and 5.1 min, respectively (Figure 1).

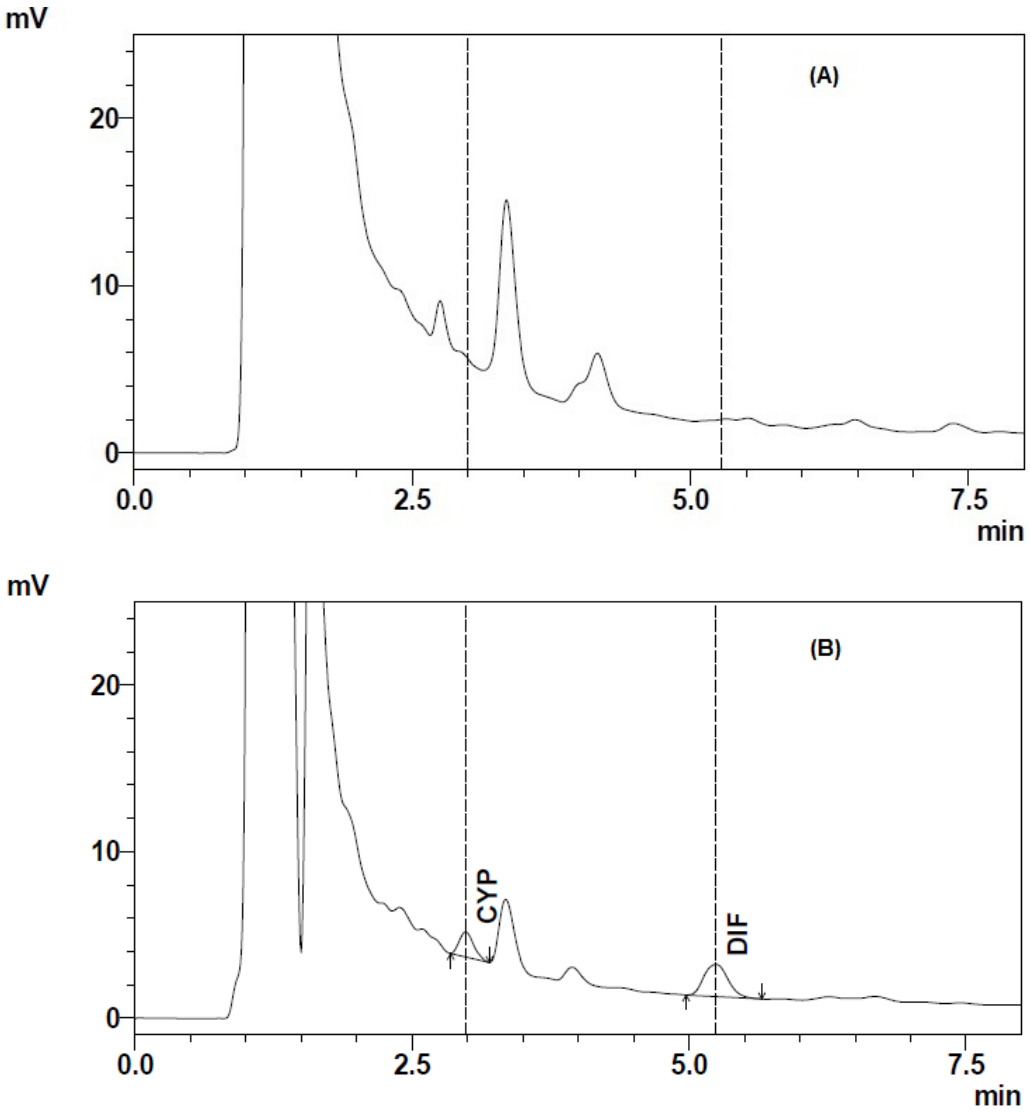
Figure 1 - Separation chromatography of the analytical standard of CYP and DIF at a concentration of 0.2 μg mL^{-1}.



HPLC-UV method validation

The method selectivity was verified by comparing a blank soil sample and a spiked blank sample. According to the chromatogram in Figure 2, the method exhibits good selectivity, and it does not interfere with the analyte retention times.

Figure 2 - Chromatograms of a blank soil sample (a) and a spiked blank sample at 0.2 mg kg^{-1} (b).



The external analytical curve was obtained from five injections at concentrations ranging from 0.1 to 1.0 μg mL^{-1}. CYP and DIF curves were submitted to ANOVA, and no deviation from linearity ($p < 0.05$) was observed. The outlier data and the homoscedasticity of the regression residuals were checked by the Huber and Cochran tests, respectively. The correlation coefficients (R) were all greater than 0.99, and the residuals were randomly distributed.

Recovery and precision tests were conducted by spiking blank samples at two levels for CYP and DIF (0.2 and 0.6 mg kg^{-1}) in five replicates. A volume of standard solution (2 μg mL^{-1} and 6 μg mL^{-1}) was added (1mL) to the sample amount (10 g), then the extraction procedure was executed. The moisture content was found to be 13.4% in the blank sample, while the values for the samples used to assess the method's applicability were 9.0%, 15.4%, and 16%, respectively. These data were used to determine the concentrations of pesticide residues in the samples. Recoveries ranged from 86% to 112% at the lower concentration level and from 71% to 118% at the higher concentration level.

The validation data for the HPLC-UV analytical method are summarized in Table 1. The concentration levels tested demonstrated recovery and precision results within acceptable parameters (Sanco, 2014).

Table 1 - Validation data of the analytical method for fungicides quantitation in soil samples.

Parameters	Cyproconazol	Difenoconazol
Linearity (R)	0.9984	0.9992
LOQ (mg kg^{-1})	0.2	0.2
Intra-day precision (CV %)		
0.2 mg kg^{-1}	9	9
0.6 mg kg^{-1}	9	14
Inter-day precision (CV% %)		
0.2 mg kg^{-1}	2	7
0.6 mg kg^{-1}	9	9
Recovery average ± SD (%)		
0.2 mg kg^{-1}	95 ± 7.7	104 ± 7.1
0.6 mg kg^{-1}	85 ± 7.3	95 ± 9.1

n=5

CYP and DIF standard solutions were stable for 24 hours at room temperature. The CYP concentration increased 8% after 15 days stored in refrigerator. Under the same conditions, the DIF concentration increased 6% at the end of the experiment (75 days). The estimate of measurement uncertainty (*U*) was calculated based on the data obtained from the method validation. The *U* values for the quantification method were ±0.08% and ±11.5% for CYP and DIF, respectively. The main contribution originated from precision tests, corresponding to 60% of CYP and 96% of DIF.

Real soil sample analysis

The applicability of the method was demonstrated through the analysis of three soil samples in triplicate. Quantitative analyses were performed using external standard calibration and quality control standards. All samples showed CYP and DIF residues below the LOQ (Table 2).

Table 2 - Results of application of the HPLC-UV validated method to real samples.

Soil sample ID	Residue level (mg kg^{-1})	
	CYP	DIF
A	<LOQ	<LOQ
B	<LOQ	<LOQ
C	<LOQ	<LOQ

ID = identification SD = standard deviation CYP = ciproconazole DIF = difenoconazole n=3 LOQ = limit of quantitation

The absence of quantifiable residues in the samples tested can be attributed to several factors. CYP and DIF may not have been applied to the crop area where the samples were collected, or, if they were applied, significant time may have elapsed between the last application and the sampling, allowing the compounds to degrade.

Pinheiro, Moraes, and Silva (2011) studied the contamination profile in soils used for onion farming. They reported a large amount of triazolic residues in the surface layer. Gerónimo *et al.* (2015) did not find these compounds in Argentine soils but detected other classes of pesticides. Scorza Júnior *et al.* (2021) monitored 46 pesticides and metabolites in the waters of the Dourados River, MS, in a region with intensive soybean and sugarcane production. Epoxiconazole (EPX) and TEB, both triazolic fungicides, were detected at high frequencies (87% and 78% of samples, respectively), with the limit of quantification ranging from 0.002 $\mu g\ L^{-1}$ to 0.02 $\mu g\ L^{-1}$.

Although the method has a limit of quantification of 0.2 mg kg^{-1}, which is higher than more sophisticated techniques. This method is thus an excellent screening tool suitable for the initial assessment of many samples or for laboratories with limited infrastructure, thus avoiding unnecessary expenses and supporting efficient laboratory workflows.

Conclusions

The development and validation of the method for screening CYP and DIF residues, two triazolic fungicides, in soils proved to be reliable and appropriate for the intended purpose. It is a low-cost method with good sensitivity and uses an accessible detector suitable for any laboratory. Although residues were not quantified in the samples analysed, which may indicate that these specific compounds were not recently used in the collection area or that they had dissipated prior to sampling, the method proved to be a low-cost tool. Therefore, it can be recommended as an alternative to processes that require the use of more robust equipment.

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