EXTRAÇÃO E DETERMINAÇÃO DE TEBUCONAZOL EM AMOSTRAS AMBIENTAIS DA CIDADE DE KARBALA NO IRAQUE E EM SUA FORMULAÇÃO USANDO CROMATOGRAFIA LÍQUIDA DE ALTO DESEMPENHO (HPLC)

EXTRACTION AND DETERMINATION OF TEBUCONAZOLE IN ENVIRONMENTAL SAMPLES FROM THE CITY OF KERBALA, IRAQ AND IN ITS FORMULATION USING HIGH- PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

استخلاص وتقدير التبيكونازول في نماذج ومستحضرات بيئية في مدينة كربلاء / العراق باستخدام تقنية الكروماتوغرافيا السائلة عالية الاداء (HPLC)

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RESUMO

O triazol, o pesticida tebuconazol, foi determinado em sua formulação e também nas amostras de água do rio coletadas de diferentes áreas agrícolas da cidade de Kerbala, no Iraque, usando o método de cromatografia líquida de alta eficiência (HPLC) desenvolvido com detecção visível por UV, fase móvel de composição era uma mistura de acetonitrila: metanol (50:50 v/v) e a coluna era C18 (250 cmx4.6mm, 5µm). Também foi utilizada a microextração dispersivo líquido-líquido modificada (DLLME), considerada um método ecológico, para a extração de tebuconazol a partir de amostras de água utilizando acetonitrila e clorofórmio como extração de solventes e agente dispersivo, respectivamente. A linearidade para manter a curva de calibração foi alcançada a partir de (0,1-70) µg·mL⁻¹ com um limite de detecção (0,053) µg·mL⁻¹ e limite de quantificação (0,174) µg·mL⁻¹. Três níveis de concentração de pico (1,0, 5,0 e 10) µg·mL⁻¹ foram utilizados para a validação do método. O desvio padrão relativo (RSD%) foi (0.294-0.813)% e a porcentagem de recuperação foi (100.001-100.005). Os estudos de formulação para duas concentrações diferentes (10 e 40) µg·mL⁻¹, preparados a partir da formulação de tebuconazol (Raxil ODS2 a 2%), proporcionam uma recuperação percentual aceitável entre (98.956-99.833). O método desenvolvido pode ser usado com precisão para a determinação de tebuconazol em amostras de água e na formulação eficaz de tebuconazol.

Palavras-chave: *Dispersivo, formulação, HPLC, apiciforme e tebuconazol.*

ABSTRACT

The triazole, tebuconazole pesticide, was determined in its formulation and also in the river water samples collected from different agriculture areas in the Iraqui city of Kerbala using developed high-performance liquid chromatography method(HPLC) with UV-visible detection, The mobile composition phase was a mixture of acetonitrile:methanol (50:50 v/v) and the column was C18 (250 cmx4.6mm,5µm). Also modified dispersive liquid-liquid microextraction (DLLME), which is regarded as an ecological -friendly method, was used for the extraction of tebuconazole from water samples using acetonitrile and chloroform as solvents extraction and dispersive agent, respectively. Linearity to maintain the calibration curve was achieved from (0.1-70) µg·mL⁻¹ with a limit of detection(0.053) µg·mL⁻¹ and limit of quantification (0.174) µg·mL⁻¹. Three spiked levels of concentration (1.0, 5.0, and 10) µg·mL⁻¹ were used for the validation of the method. The relative standard deviation (RSD%) was (0.294-0.813)%, and the percentage recovery was (100.001-100.005). The formulation studies for two different concentrations (10 and 40) µg·mL⁻¹, which prepared from tebuconazole formulation (Raxil ODS2 2%), gave acceptable percentage recovery between (98.956-99.833). The developed method can be used accurately for the

determination of tebuconazole in water samples and in the formulation of tebuconazole effectively.

Keywords: Dispersive, formulation, HPLC, spiked, and tebuconazole.

الملخص

الترابازول , مبيد التبيكونازول, تم تقديره في محاليله وكذلك في نماذج من مياه النهر جمعت من مناطق زراعية مختلفة في مدينة كربلاء / العراق باستخدام تقنية كروماتوغرافيا السائلة عالية الأداء وباستخدام الكاشف في المنطقة المرئية وفوق البنفسجية . تركيب الطور المتحرك هو عبارة عن مزيج من الاسيتونتريل : ميثانول (50:50 حجم/حجم) والكولوم المستعمل من نوع C18 بابعاد (250 سم x 4.6 ملم x 5 مايكرو ملم) . وكذلك تم استخدام طريقة تشتت سائل-سائل المايكروية, التي تعتبر من طرق الاستخلاص الصديقة للبيئة, لاستخلاص التبيكونازول من نماذج الماء باستخدام الاسيتونتريل والكوروفورم كمحاليل للاستخلاص والتشتت . الخطية لمنحني المعايرة تم الوصول إليها عند تراكيز (0.1-70) ملي مايكرو/ مل وكان حد الكشف (0.053) ملي مايكرو/ مل وحد الكشف الكمي (0.174) ملي مايكرو/ مل. ثلاث تراكيز (5.0, 10.0 و 10.0) مايكرو غرام/ مل تم استعمالها لتقييم الطريقة . الانحراف المعياري النسبي كان (0.294-0.813) ونسبة الاسترجاع كانت (100.001-100.005). تمت دراسة المحاليل للتبيكونازول لتركيزين (10 و 40) ملي مايكرو/ مل , والتي حضرت من محاليل التبيكونازول (راكسيل 2% ODS₂) وأعطت نسب استرجاع بين (98.956-99.833). الطريقة المحدثة يمكن تطبيقها بدقة لتقدير التبيكونازول في نماذج الماء وكذلك في محاليل التبيكونازول بكفاءة .

الكلمات المفتاحية: تشتت , محاليل , HPLC , spiked , تبيكونازول .

1. INTRODUCTION

Triazole was, synthesized by Fisher in 1878, consist of three nitrogen atoms in positions 1,2, and 4 forming five membranes heterocyclic ring, its weak properties made it soluble in all organic solvent. Triazoles have many applications in agriculture, medicine and industry when fused with pyrimidines, triazine, pyridazine and pyrazine. (Maddila et al., 2013). Hydrocarbon backbone in triazole bounded with nitrogen in position1. The chiral center, due to the presence of nitrogen in the triazole ring, gives this compound its bioactivity when it is used as fungicides against fungal disease in different crops (SHAH et al., 2006; Subova et al., 2006). Generally, all formulations of triazole were found as a racemic mixture, thus gives triazole enantioselectivity phenomena, Although there is an exception in some of the triazole pesticides, which consist of one enantiomer, such as uniconazole and diniconazole (Wang et al., 2010).

Triazole regarded as the most interesting heterocyclic compounds that inter in the constituent of cell and living matter (Palmer et al., 2012).

Tebuconazole (TEB) (figure 1), (R,S)-1-*p*-chlorophenyl-4,4-dimethyl-3-(1*H*-1,2,4-triazol-1-ylmethyl)pentan-3-ol, is a triazole fungicide used to control different plant diseases but has a toxic effect on the organisms in the water environment (Yu et al., 2013).

Figure 1: Chemical structure of tebuconazole

Risk assessment for the triazole fungicides became necessary due to the toxicological properties of tebuconazole especially in children and farmers through the ingestion of contaminated food and also due to the inappropriate treatment done without following safety instruction leading to remaining the residue in food after harvest (Liu, 2017, Lutsevich et al., 1995, Oliva et al., 2007).

Various methods were reported for the determination of tebuconazole to monitor its residues in the different environmental samples. These methods includes gas-liquid chromatography (Patil et al., 1992, Vorpsi et al., 2011, Farajzadeh et al., 2014), Liquid chromatography-mass spectrophotometry (Caldas et al., 2011, Khalil and Huat, 2013, Kundu et al., 2011), gas chromatography-mass spectrophotometry (Liu et al., 2011, Dong and Hu, 2014), High-performance liquid chromatography (Rao et al., 2012, Caldas et al., 2009).

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Tebuconazole standard (purity > 99%) was supplied by Dr. Ehrenstorfer GmbH company. Water, Acetonitrile, and methanol for HPLC analysis with purity (> 99.9%) were purchased from the Sigma-Aldrich company. Tebuconazole Formulation (Raxil ODS₂ 2%) was obtained from Karbala Agriculture Department. Orthophosphoric acid (85%) was provided from Merck (Darmstadt, Germany), hydrochloric acid (36w/v%) from HIAMEDIA, and Sodium hydroxide (99%) from B.H.D company.

2.1.1. Preparation of Formulation Solution

One milliliter from tebuconazole formulation (Raxil ODS₂ 2%), obtained from Karbala Agriculture Department, was diluted with 100 mL methanol, then different volumes were taken for this solution to prepare different concentration of tebuconazole for HPLC analysis. All solutions were kept in 4°C.

2.1.2. Preparation of Standards Solutions

The calculated amount of standard tebuconazole was diluted with methanol to prepare (1000 µg·mL⁻¹). Series solutions from the stock solution ranged from (0.5 - 200) µg·mL⁻¹ were prepared in 5 mL volumetric flask for studying the calibration curve. All solutions were kept in 4°C.

2.1.3. preparation of sodium hydroxide (0.1 M)

Sodium hydroxide (NaOH) was prepared by dissolving 0.4 g of sodium hydroxide in 100 mL distilled water.

2.1.4. preparation of orthophosphoric acid (0.1M)

Orthophosphoric acid (H₃PO₄) was prepared by diluting 0.680 mL of concentrated orthophosphoric acid in 100 mL distilled water.

2.1.6. Modified Dispersive liquid-liquid microextraction method (DLLME)

Five milliliters of water samples, collected from different agriculture areas in Kerbala city/ Iraq, were filtered and the filtrates were transferred to a glass centrifuged test tube (10mL) with a screw and spiked with different concentrations of standard tebuconazole (1.0, 5.0 and 10) µg·mL⁻¹. Sodium chloride (5% v/v, 0.5 mL) was added as

salting-out agent. A mixture of acetonitrile (1 mL) and chloroform (100 µL) was added to the spiked samples as solvent extraction and dispersant agent, respectively. The final solution was vortexed for 2 minutes, followed by shaking by hand for 10 minutes, then centrifuged at 5000 rpm for 15 minutes. Two layers were separated. The aqueous layer removed by microsyringe and the organic layers, which contain tebuconazole as droplets, were diluted with 0.5 mL of acetonitrile for HPLC analysis injection after filtration through Millipore filter paper 0.45 µm. The same procedure was applied for river water samples collected from different agriculture areas from Kerbal city / Iraq without spiking as a controller.

2.2. INSTRUMENTATION

Double beam UV-spectrophotometer-1800, Shimadzu. Digital balance, Denver-TP-214. High-performance liquid chromatography (HPLC) 20 AD Shimadzu equipped with UV-detector. Ultrasonic cleaner, KQ200E. pH-meter, Hanna-pH211. Centrifuge, C2 series.

2.3. METHOD VALIDATION

2.3.1. Chromatographic conditions

High-performance liquid chromatography system (LC-20AD, SHIMADZU Corporation) equipped with an autosampler, hyper sail ODS C18 column (25cmx4.6mm, 5µm), and UV-Visible detector was used for analysis. The composition of the mobile phase was acetonitrile:methanol (50:50 v/v) at pH 5.0 using H₃PO₄/NaOH (0.1M) for adjusting the pH. The flow rate was 0.8 mL/min, and the volume of injection was 20 µL at λ_{max} 220 nm.

2.3.2. Wavelength selection

Standard tebuconazole solutions (50 and 10) µg·mL⁻¹, prepared in methanol from stock solution, were scanned from 190-800 nm. It was found there are two peaks that appear, low-intensity peak at λ_{max} 268 and high-intensity peak at λ_{max} 220 nm (figure 2). The peak at 220 nm was chosen for optimization due to its high intensity.

2.3.3. Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limit of detection (LOD) is the lowest

concentration of analyte detected by the proposed method but may not be quantified, on the other hand, the limit of quantification(LOQ) is the lowest concentration of analyte that can be detected quantitatively with high precision and accuracy. LOD and LOQ measured due to the ratio of noise(N) to signal(S) of the baseline. LOD calculated if the ratio (N/S=3), while LOQ calculated when the ratio (N/S=10).

2.3.4. Precision and Accuracy

The precision of results in the proposed method represented by the repeatability of injection samples (n=5) and measured by relative standard deviation value (RSD%), while recovery percentage represents accuracy. Different concentrations (0.5,10 and 50) $\mu\text{g}\cdot\text{mL}^{-1}$ from tebuconazole standard were prepared and tested for validation study by injection in HPLC devise under optimum conditions(n=5).

3. RESULTS AND DISCUSSION

3.1. Method development

Some preliminary investigations were performed, which included tested two types of columns (C18 and C8) to perform the best response and acceptable peak shape and capacity. It was found that the best separation was manyained by using C18 column rather than C8 coolumn. Also, different ratios of solvents (methanol, acetonitrile, and water) with different compositions ranged from (90:10 v/v) to (50:50 v/v) were tested for the best separation. Mobile phase with composition of acetonitrile:methanol (50:50 v/v) was chosen for optimization according to the highest response, good peak shape and acceptable capacity factore and resolution (figure 3) . All preliminary investigations were maintained at flow rate $1\text{mL}\cdot\text{min}^{-1}$ and the injection volume of $20\mu\text{L}$ at $\lambda_{\text{max}}220\text{nm}$.

3.2. Method validation

3.2.1. Effect of pH

The mobile phase with different pH ranging from (3.0-8.0) were studied for the best response, tebuconazole ($50\mu\text{g}\cdot\text{mL}^{-1}$) was injected, and the pH justified by using NaOH/phosphoric acid (0.1M).

Table 1. Effect of pH.

pH	Retention time (tR) (min.)	Peak area	Capacity (k)	Tailing factor (TF)
3.0	2.883	1825538	0.765	1.166
4.0	2.773	1802392	0.864	1.079
5.0	2.722	1872780	0.935	1.004
6.0	2.731	1776323	0.903	1.021
7.0	2.763	1755021	0.934	0.951
8.0	2.902	1011624	0.972	1.162

Results illustrated in Table 1 and figure 4 proved that pH 5.0 was the best choice according to the good resolution, acceptable peak shape and capacity, for that pH 5.0 was used for optimization.

3.2.2. Effect of flow rate

At pH 5.0, tebuconazole with concentrarion ($50\mu\text{g}\cdot\text{mL}^{-1}$) was injected (n=3) at various flow rate ranged from (0.3 -1.5) mL/min.

Table 2. Effect of flow rate.

Flow rate mL/min	Retention time(tR) (min.)	Peak area	Capacity (k)	Tailing factor (TF)
0.5	5.372	3511399	0.974	1.211
0.8	3.315	2293504	0.981	1.024
1.0	2.662	1864464	0.894	1.223
1.3	2.221	1473194	0.921	1.332
1.5	1.782	1181085	0.916	1.361

From results in table 2 and figure 5, the highest response obtained was at flow rate $0.5\text{ mL}\cdot\text{min}^{-1}$,but at this flow rate an acceptable vaule of tailing factor obtained. Flow rate at $0.8\text{ mL}/\text{min}$ was adopted for optimization as a result of maintaining good symmetric shape, acceptable capacity, and short retention time (Table 2).

3.2.3. Effect of volumes injection

Different volumes eanged from (5.0-25.0 μL) of tebuconazole standard of concentration ($50\mu\text{g}\cdot\text{mL}^{-1}$) were injected(n=3) to HPLC system at optimum conditions. The results explained in table 3 and figure 6.

Table 3. Effect of volume injection.

Volume (μL)	Retention time(t _R) (min.)	Peak area	Capacity (k)	Tailing factor (TF)
5	3.313	520218	1.028	2.155
10	3.318	881235	0.990	1.394
15	3.328	1656336	0.937	1.280
20	3.331	2282035	0.987	1.086
25	3.335	2850382	0.906	1.277

Result in Table 3 and figure 6 explained that after injection the volume of 20 μL the tailing factor was increased and an acceptable peak shape was obtained due to overloaded of the column which affected the shape of peak and baseline stability, while up to 20 μL the responses were increased systematically with improvement in peak shape, for that the volume 20 μL was chosen for optimization.

3.2.4. Linearity

For linearity study, series solutions ranged from (0.1-100) μg·mL⁻¹ were prepared from the stock solution of standard tebuconazole and injected (n=3) into the HPLC system. The calibration curve was maintained by plotting peaks area against concentrations (figure 7). All solutions were filtered through a 0.45 μm Millipore filter.

Table 4. Analytical parameter for the Tebuconazole calibration curve.

λ max (nm)	220
Linearity range (μg mL ⁻¹)	0.1-70
Limit of detection (μg mL ⁻¹)	0.072
Limit of quantification (μg mL ⁻¹)	0.240
Regression Equation	y = 60592x + 126273
Slope	60592
Correlation coefficient (R ²)	0.9997

Linearity was achieved at concentration ranged from (0.1-70) μg mL⁻¹ after that deviation observed from Lambert's law with correlation coefficient (0.9997). The limit of detection and limit of quantification are 0.072 and 0.240 μg mL⁻¹, respectively (table 4).

3.2.5. Precision and Accuracy

At optimum conditions, three concentration

levels of tebuconazole (0.5, 10 and 50) μg·mL⁻¹ were injected (n=5). The results sorted in Table 5.

Table 5. Precision and Accuracy for the modified method.

Conc. (μg·mL ⁻¹)	Retention time(t _R) (min.)	Recovery %	RSD %
0.5	3.331	100.007	0.929
10.0	3.330	100.000	0.045
50.0	3.331	100.002	0.453

From results in Table 5, good recovery (100.000-100.007) and low RSD% values (0.045-0.929) were obtained, for that this method was accurate to determine the amount of tebuconazole in environmental samples.

3.3. Application

The optimized method was applied for the determination of tebuconazole in spiked river water samples (1.0, 5.0, and 10) μg·mL⁻¹ collected from different agriculture areas in Kerbala City / Iraq and in its formulation (Raxil 2%) (Tables 6-7) and (figures 8-10).

Table 6. Recovery of Tebuconazole in water samples spiked with three different levels.

Spiked level (μg·mL ⁻¹)	Amount found (μg·mL ⁻¹)	Recovery % (n=5)
0	Not detected	-
1.0	0.839	83.184
5.0	4.546	90.904
10.0	9.659	96.594

Table 7. Recovery of Tebuconazole in the formulation.

Formulation (μg·mL ⁻¹)	Amount found (μg·mL ⁻¹)	Recovery % (n=5)
10.0	9.892	98.956
30.0	29.250	97.502

Result in table 6 explained that there is no detection for tebuconazole was obtained for water samples collected from different agriculture area due to the very low concentration of tebuconazole in these samples. For spiked samples the percentage recoveries were ranged from (83.184-96.594) while the percentage recoveries for different prepared concentration of formulation

(table 7) were ranged from (97.503-98.956).

4. CONCLUSION

The sensitive, selective, and accurate method was developed to extract and determine the tebuconazole in the water sample. Tebuconazole residues in six samples collected from different agriculture area in Kerbala City / Iraq was not detected due to the very low concentrations of the residues in water samples. Good recoveries and relative standard deviations values were obtained which made the proposed method can be applied successfully for the determination of tebuconazole in environmental samples and in its formulations.

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Figure 2: UV-visible spectra of Tebuconazole

Figure 3. Chromatogram of standard Tebeconazole

Figure 4. Effect of pH

Figure 5. Effect of Flow rate

Figure 6. Effect of volume injection

Figure 7. Calibration curve for α-Cypermethrin standards

Figure 8. Chromatogram of spiked water sample(1.0) $\mu\text{L mL}^{-1}$

Figure 9. Chromatogram of spiked water sample(5.0) $\mu\text{L mL}^{-1}$

Figure 10. Chromatogram of spiked water sample(10.0) $\mu\text{L mL}^{-1}$