HPLC METHOD FOR DETERMINATION OF ACTIVE INGREDIENTS IN PESTICIDE FORMULATION SWITCH 62,5 WG

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ABSTRACT

Analytical quality control for pesticides achieved using appropriate methods is essential in providing their safe and adequate use in agriculture. This study presents a precise, fast, and a simple analytical method for determining active ingredients cyprodinil and fludioxonil in a fungicide formulation Switch 62,5 WG using high-performance liquid chromatography (HPLC). The development of the HPLC method was performed on a ZORBAX Eclipse Plus C18 (50 x 4.6 mm, 1.8 μm) analytical column using isocratic elution with a mobile phase consisting of acetonitrile and water in volume ratio 70:30, a flow rate of 1 mL/min, a constant column temperature at 25 ºC and UV detection at 220 and 270 nm. The run time of analysis under these chromatographic conditions was about 1.5 min. The method was validated by testing specificity, linearity, precision, recovery, LOD, LOQ, and accuracy according to the CIPAC (Collaborative International Pesticides Analytical Council) and SANCO (Directorate General Health and Consumer Protection) guidelines, and all the tested parameters were found within acceptance criteria. The values for multiple correlation coefficient ($R^2 \geq 0.99$), relative standard deviation (RSD < 1 %), recoveries ranged from 98.95 - 102.26 %, revealed that the developed method has a good linearity, precision and accuracy. The proposed method is suitable for routine analysis of active ingredients cyprodinil and fludioxonil in the formulation Switch 62,5 WG.

Keywords: cyprodinil, fludioxonil, HPLC method, pesticides, fungicide formulation.

INTRODUCTION

Pesticides are intended to increase crop production in agricultural farms, reduce or eliminate yield losses and maintain high product quality. Public concern over the use of pesticides has emerged as an essential issue in agricultural safety. Analytical quality control for pesticides achieved using appropriate methods is necessary for providing their safe and adequate use in agriculture (Joint FAO/IAEA, 2009). Accurate and precise analytical methods are required to generate data for authorization and post-registration control and monitoring purposes under Regulation (EC) No1107/2009 (2009). The improvement of existing and the development of new analytical methods for the determination of active substances in plant protection products are necessary to control the quality of pesticide formulations.

Switch 62,5 WG is a fungicide formulation that CIPAC (2017) classifies as pesticides in the form of water-dispersible granules. This fungicide contains two highly effective active ingredients: cyprodinil and fludioxonil with combined contact and systemic properties to control *Botrytis* (Pest Management Regulatory Agency, 2006).

Cyprodinil, the systemic component, is anilinopyrimidine, whose mechanism of action consists of the inhibition of methionine biosynthesis (Masner et al. 1994). Cyprodinil (Figure 1 a) is a generally accepted name according to the International Organization for

Standardization (ISO) (FAO, 2009), while according to IUPAC the name of this active component is 4-cyclopropyl-6-methyl-N-phenylpyrimidin-2-amine (Shah, 2003). This pyrimidinamine fungicide was developed and introduced by Novartis Crop Protection AG and was first marketed in 1994 (Lui et al., 2011). Solubility is good in water, ethanol, acetone, toluene, *n*-hexane, *n*-octanol (MacBean, 2008 a).
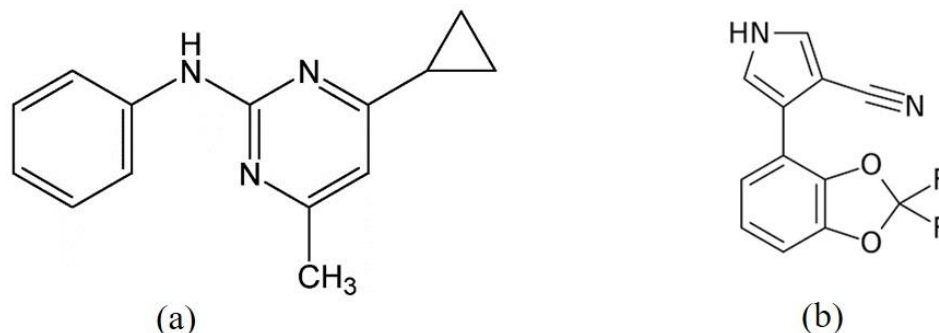

Figure 1. Chemical structure of cyprodinil (a) and fludioxonil (b)

Fludioxonil (Figure 1 b) is a generally accepted name according to the International Organization for Standardization, while according to IUPAC, the name of this active component is 4-(2,2-difluoro-1,3-benzodioxol-4yl)pyrrole-3-carbonile (Tomlin, 1997). Fludioxonil, a phenylpyrrole, is a nonsystemic fungicide, affecting the plasmatic membrane's transportation processes (Jespers et al., 1993). These active ingredients have been approved for use according to the Regulation (EC) No 1107/2009 (2009). Solubility is good in water, ethanol, acetone, toluene, *n*-hexane, *n*-octanol (MacBean, 2008 b).

The literature search shows that various analytical methods have been reported for the quantitative determination of cyprodinil and fludioxonil residues in a variety of fruits, vegetables, and food commodities. For example, Fernandez et al. (2008) described a liquid chromatography method to determine cyprodinil and fludioxonil in the fermentative process of must. Pan et al. (2009) analysed the cyprodinil residues in grape using gas-chromatography (GC). GC coupled with mass spectrometry and HPLC coupled with mass spectrometry was also introduced to determine cyprodinil (Patil et al. 2009; Romero-Gonzalezet et al. 2008). Schummer et al. (2012) developed a highly sensitive method based on solid-phase microextraction and gas chromatography-tandem (triple quadrupole) mass spectrometry to test hair samples for 50 pesticides. The results showed that the concentration in volunteers' hair matched with agricultural activity, and the highest concentration was observed exactly for cyprodinil (1161 pg/mg). The residue of fludioxonil was quantitatively analysed in blueberries (Munitz et al., 2013) and grape and lettuce (Marín et al., 2003) by GC-NPD. Mercader et al. (2014) applied ELISA technique for fludioxonil determination in fruit juices. Lazić et al. (2016) described method for cyprodinil and fludioxonil fungicide residues and dissipation in lettuce. Fungicides extraction from lettuce was performed by QuEChERS method, while determination was accomplished by HPLC-DAD using C18 column.

However, few studies were related to the analysis of active substances in the pesticide formulation and their quality control. To the best of our knowledge, no liquid chromatographic methods for determination of cyprodinil and fludioxonil in pesticide formulations have been published in the literature. Hence, the aim of this study was to develop a new, precise, fast, and simple analytical method for determination of active ingredients cyprodinil and fludioxonil in a fungicide formulation Switch 62,5 WG using high-performance liquid chromatography (HPLC) and ultraviolet diode-array detection (UV-DAD).

MATERIALS AND METHODS

Reagents and chemicals

The analytical standards of cyprodinil with a purity of 99.9 % and fludioxonil with a purity of 99.8 % purchased by Syngenta (Bazel, Switzerland), were used for the preparation of stock and working solutions. Ultrapure water was produced by TKA Smart2 Pure 13 UV/UF water purification system (Germany). HPLC-grade acetonitrile (99.9 %) and methanol (99.8 %) and filter membranes with pore size 0.45 μm Iso-Disc PTFE Supelco were purchased from Sigma-Aldrich (Germany). The pesticide formulation Switch 62,5 in the form of water-dispersible granules (WG) was produced by Syngenta (Bazel, Switzerland). The declared value for the content of cyprodinil was 37.5 % and 25 % for fludioxonil.

Equipment

The chromatographic analysis was performed on an Agilent 1260 Infinity Rapid Resolution Liquid Chromatography (RRLC) system equipped with: vacuum degasser (G1322A), a binary pump (G1312B), an autosampler (G1329B), a thermostatted column compartment (G1316A), and a UV-VIS diode array detector (G1316B). Data acquisition was performed on ChemStation software (version C.01.02). For better dissolving of the stock solutions, an ultrasonic bath "Elma" was used. The investigations were carried out on a ZORBAX Eclipse Plus C18 (50 x 4.6 mm, 1.8 μm, Agilent Technologies) analytical column.

Preparation of Standard Solutions

Stock solution for fludioxonil was prepared by dissolving 0.0052 g of the pure analytical standard with methanol in a 10 mL volumetric flask. Accurately weighed 0.0081 g cyprodinil standard was dissolved in 10 mL volumetric flask with methanol. The prepared stock solutions were ultrasonicated for 15 min and stored in a refrigerator at 4 °C.

Stock solutions were used to prepare a series of 6 working solutions containing both active ingredients with a concentrations of 16.42, 32.84, 43.79, 58.38, 77.84 and 103.79 μg/mL for fludioxonil and 25.60, 51.21, 68.28, 91.03, 121.38, and 161.84 μg/mL for cyprodinil in 10 mL volumetric flask by dilution with the mixture of acetonitrile/water (70/30, *V/V*). These working solutions were prepared for determination of the linearity of the method. Each of these working solutions was injected three times with a volume of 5 μL.

Preparation of Sample Solution

Sample solution of pesticide formulation Switch was prepared in a 10 mL volumetric flask by dissolving the weighed amount of 0.0305 g in methanol. The prepared solution was ultrasonicated for 15 min. From this sample solution, 0.5 mL was transferred to a 10 mL volumetric flask and dissolved with the mixture of acetonitrile/water (70/30, *V/V*). Five injections were performed with a volume of 5 μL of this solution.

To determine the accuracy of the method in three flasks of 10 mL, 0.5 mL of the prepared sample solution was taken. A known quantity of analytical standards was added to each solution and diluted to volume with the same solvent mixture. 5 μL of each of these solutions were injected five times.

Before HPLC analysis, all the solutions were filtered through a 0.45 μm PTFE Iso-Disk filter.

RESULTS AND DISCUSSION

Development of a new analytical method for simultaneously determination of active ingredients cyprodinil and fludioxonil in a pesticide formulation Switch 62,5 WG was accomplished using HPLC and UV-DAD. Separation of analytes was achieved on a ZORBAX

Eclipse Plus C18 (50 x 4.6 mm, 1.8 μm) analytical column. Eclipse Plus C18 columns are designed for high efficiency and excellent peak shape with all sample types. They are especially useful for separating acidic, basic, and other highly polar compounds by reverse-phase liquid chromatography – RP-LC (Agilent technologies, 2006). It was found that the optimum separation and symmetrical peak shape of the investigated pesticide were achieved with mobile phase consisted of acetonitrile/water (70/30, *V/V*), isocratic elution with a flow rate of 1.0 mL/min, constant column temperature at 25 ℃, and UV detection performed at 220 nm and 270 nm. Under these chromatographic conditions, a smooth baseline was obtained, and the chromatographic peaks of cyprodinil and fludioxonil were high, narrow, and symmetrical (Figure 2 and Figure 3). The obtained values for column dead time was 0.20 min, the fludioxonil retention time was 0.79 min, and retention time of cyprodinil was 1.15. Consequently, the calculated values for the retention factor (*k'*) were 2.95 for fludioxonil and 4.75 for cyprodinil. These values belong to the range of optimal values for this parameter (Dong, 2006). The run time of analysis was about 1.5 min. The short run time means that for this analysis a small volume of organic solvent required, making this method cost-effective and environmental friendly.
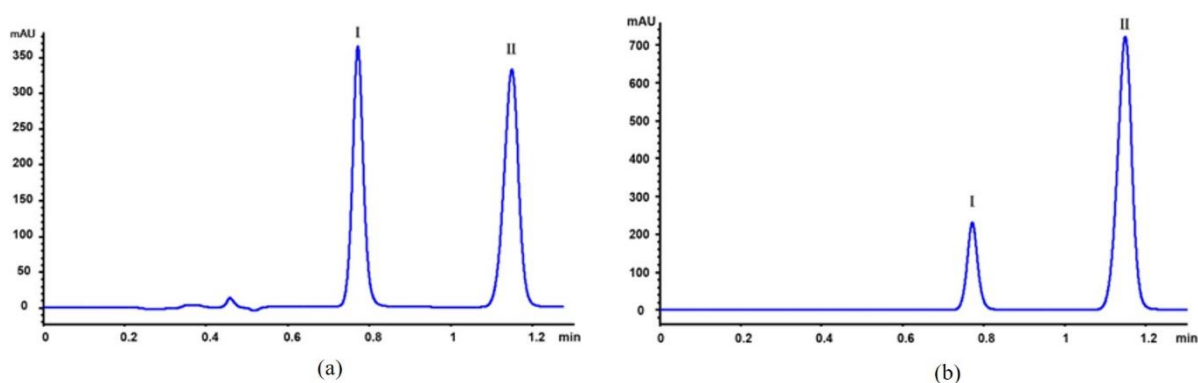

Figure 2. Chromatograms obtained from the analytical standard of fludioxonil (I) and cyprodinil (II) at UV detection of 220 nm (a) and 270 nm (b)
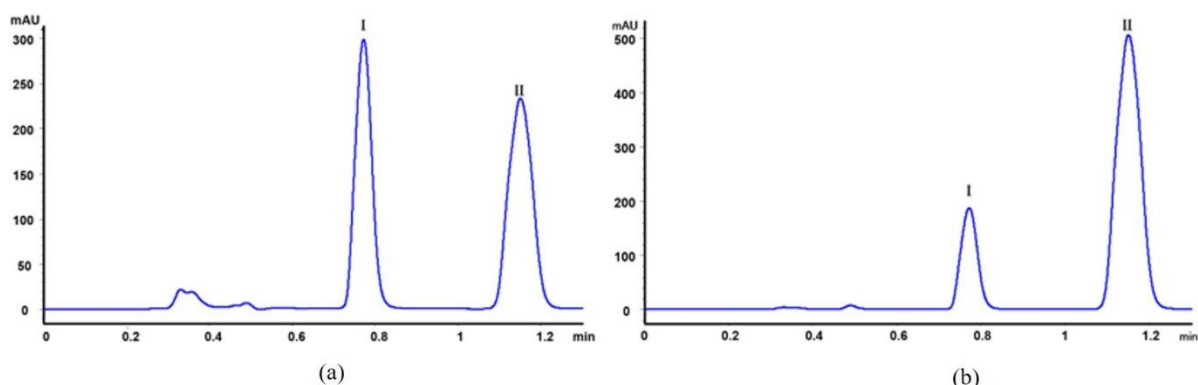

Figure 3. Chromatograms obtained from pesticide formulation Switch 62,5 WG at UV detection of 220 nm (a) and 270 nm (b)

From the obtained chromatograms (Figure 2 and Figure 3), we have noticed that the peak for the active ingredient fludioxonil (I) was higher at UV detection of 220nm (a), and the peak for cyprodinil (II) was higher at UV detection of 270nm (b).

In order to establish the appropriateness of the method for its future application, the developed method was validated by testing specificity, linearity, precision, recovery, LOD,

LOQ, and accuracy in accordance with the CIPAC (2003) and SANCO (European Commission, 2019) rules.

In addition, to confirm the specificity and selectivity of the developed method, the UV diode array detection was used to check the peak purity and analyte peak identity. The specificity and selectivity of the developed method were estimated by identifying the peak of interest and value for the index of peak purity. The analyte identification was performed by comparing its retention time in the standard solution and the sample and confirmed by overlaid spectra of pure analytical standard of the active substance and the absorption spectra of the same substance in pesticide formulation (Jenkie, 1996). As can be seen from the chromatograms of the pesticide formulation (Figure 3), besides the chromatographic peaks of the active ingredients, there were no other coeluted peaks that interfere. Moreover, the value of the match factor obtained by overlaid spectra (Figure 4) was 999.971 (for fludioxonil) and 999.958 (for cyprodinil), indicating that the peaks were of the same substances.
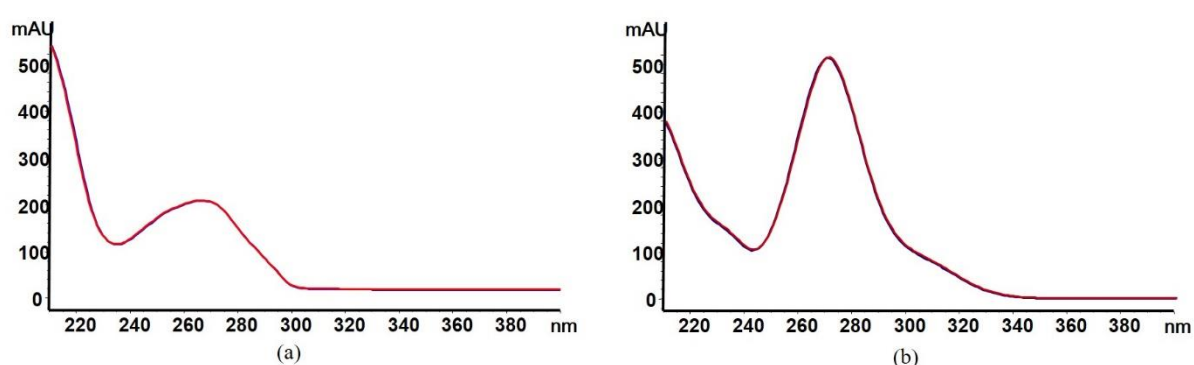

Figure 4. The overlaid UV spectra obtained by comparing the absorption of a pure analytical standard of of active ingredient absorption spectra of the same analyte in the pesticide formulation Switch 62,5 WG for fludioxonil (a) and cyprodinil (b)

The linearity of the method was tested by constructing calibration curves that give the dependence of the peak area and the peak height on the injected amount of analyte. For this purpose, a series of 6 working solutions of different concentrations in the concentration range of 16.42 - 103.79 µg/mL for fludioxonil and 25.60 - 161.84 µg/mL for cyprodinil were prepared. The obtained results for multiple correlation coefficients ($R^2 \geq 0.99$) indicated that the method has an excellent linearity. The results for multiple correlation coefficients given in Table 1 indicated preferably the use of peak area at UV detection of 270 nm as a variable.

Table 1. Results for the linearity of the method

		Linearity range (µg/mL)	Regression equation	R^2
fludioxonil	220 nm	164.2 – 103.79	[1]y = 20.939x + 94.283	0.9962
			[2]y= 9.3267x + 62.347	0.9893
	270 nm		[1]y = 12.964x + 4.6188	0.9998
			[2]y = 6.1397x + 29.978	0.9919
cyprodinil	220 nm	256 – 161.84	[1]y = 16.241x + 24.216	0.9994
			[2]y = 5.344x + 61.349	0.9837
	270 nm		[1]y = 34.809x + 42.63	0.9997
			[2]y = 11.477x + 138.81	0.9824

[1]y – peak area
[2]y – peak height

Precision was expressed as repeatability of obtained results, which was evaluated for retention time and peak area of cyprodinil and fludioxonil from 10 successive injections of mixture of analytical standards with a concentration of 51.21 μg/mL for cyprodinil and 32.84 μg/mL for fludioxonil. The results were tested according to the criteria laid down in CIPAC Document 3807 (2003). The RSD values for retention time (1.20 % for cyprodinil and 0.99 % for fludioxonil) and relative standard deviation (RSD) of peak area (0.98 % for cyprodinil and 0.97 % for fludioxonil) indicated a very good precision of the tested method.

The limit of detection (LOD) was defined as the amount of analyte for which the signal to noise ratio (S/N) was three, whereas the limit of quantification (LOQ) was defined as the amount of analyte for which S/N = 10 (Lough & Wainer, 1996). The obtained LOD values were 1.12 ng for cyprodinil and 3.08 ng for fludioxonil, while, the LOQ values were 4.0 ng for cyprodinil and 10.16 ng for fludioxonil.

The method of standard additions was used for determination of accuracy of the developed method (CIPAC, 2003). Accuracy of the method was expressed as the deviation between the calculated mean value obtained by examination and the actual value of the spiked amounts of the analytes into a sample matrix that already contains some quantity of the analytes (Table 2). As shown in Table 2, the obtained values for recovery were within the following ranges (98.95 – 102.26 %), which were in accordance with CIPAC criteria (CIPAC, 2003). Consequently, it was concluded that the proposed method was accurate enough for the determination of active ingredients in the pesticide formulations.

Table 2. Results for recovery ($n = 5$)

	m(analyte) before addition (μg)	m(analyte) added (μg)	m(analyte) after addition (μg) ($\pm$SD)	Recovery (%)	RSD (%)
fludioxonil	398.21	93.41	496.65 $\pm$ 2.82	101.02	0.57
		186.83	594.67 $\pm$ 4.58	101.65	0.77
		280.24	693.77 $\pm$ 2.47	102.26	0.36
cyprodinil	573.21	145.65	711.34 $\pm$ 4.77	98.95	0.67
		291.31	863.16 $\pm$ 1.10	99.84	0.13
		436.96	1002.06 $\pm$ 0.67	99.20	0.07

The developed HPLC method was successfully applied for determination of the content of active ingredients in analysed pesticide formulation Switch 62.5 WG. The mean obtained value for fludioxonil was 26.11 % and for cyprodinil was 37.59 %. These values corresponded to that declared by the manufacturer.

CONCLUSION

A precise, fast, simple and accurate HPLC method with UV-DAD for simultaneous determination of active ingredients cyprodinil and fludioxonil in a fungicide formulation Switch 62,5 WG has been developed and validated. The analysis was performed on a ZORBAX Eclipse Plus C18 (50 x 4.6 mm; 1.8 μm) analytical column. The proposed method showed a high values of multiple correlation coefficients for calibration equations and repeatability of retention time and peak area. According to the CIPAC and SANCO guidelines, all the tested parameters were found within acceptance criteria.

This method requires a small volume of organic solvent, making it environmentally friendly and cost-effective. The proposed method is suitable for routine analysis of active ingredients cyprodinil and fludioxonil in the formulation Switch 62,5 WG. Also, the method can be applied to the analysis of these active substances in other matrices, of course by prior testing in order to determine if some modification is required.

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