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Quantification and risk assessment of Flutianil and of propiconazole in green bean by HPLC-DAD under greenhouse conditions

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Abstract

Green bean (*Phaseolus vulgaris* L.) is one of the important cash crops; which is subjected to many pests, consequently pesticides are used. Flutianil as a novel compound and propiconazole as a reference compound, were sprayed at the recommended dose on green bean. Their residues and safety to humans under greenhouse conditions were evaluated. Samples were randomly collected at (2 h), 1, 3, 5, 7, 10, 15 and 21 days, respectively. Extraction was performed applying a quick, easy, cheap, effective, rugged, and safe approach (QuEChERS) to homogenized samples, coupled with HPLC-DAD for residues determination. Method was validated starting with blank samples, spiked at five levels (n = 6). Linearity was assessed by injections in triples (n=3) for five concentrations (0.01 to 10 mg kg -1 each), resulting in good linearity with regression coefficient (R^2) 0.9956 and 0.9999, high accuracy, precision, matrix effect, satisfactory recoveries (76.9%–110.2%) and relative standard deviation (<20%). The limits of detection and quantification were 0.01 and 0.05 µg/kg, respectively. Pre-harvest intervals (PHIs) were 10 days and maximum residue limits (EU MRLs) was 0.01 for both pesticides. The assessment of health risk, based on dietary exposure, showed that green beans treated with both pesticides are safe to human.

Keywords: Green beans; Flutianil; propiconazole; residues; HPLC; risk assessment.

1. Introduction

Legumes are considered as a significant source of plant-based protein for human dietary all over the world [1]. The beans are of multi- uses to consumers as vegetable pods, dried seeds, besides their use as animal feed [2]. In Egypt the green bean (*Phaseolus vulgaris*), is one of the most important food and cash crops, significant amounts are annually exported to Europe [3]. The production fluctuated through 1968 - 2022 period. In 2020 the green bean production reached 264,959, tones with an export value of USD 30008k and in 2021 the export volume was 1.38M metric ton and USD 1.46M export value [4-5]. The yield of many agricultural crops is severely reduced due to infestation by pests and diseases [6]. Powdery mildew, red spider mite, leaf miners, aphids, pod borers, and greasy cutworms are among the pests and diseases that attack green beans and cause a 12-30% yield loss [7--10]. Powdery mildew is a major production problem which reduces yields by decreasing the size or number of pods, their quality, and may cause plants to die or damage pods severely [11-12].

To control pests, decrease the loss in yield and enhance crop production, many pesticides are used, among which are Flutianil and Propiconazole. Flutianil ($C_{19}H_{14}F_4N_2OS_2$) is a novel thiazolidine antifungal fungicide that is protective, curative, and translaminar against powdery mildew at low dosages on various crops. It shows no cross-resistance and prevents disease expansion. Propiconazole ($C_{15}H_{17}C_{l2}N_3O_2$) is a broad spectrum foliar triazole with systemic properties for the control of powdery mildew, rusts, and leaf spot. It is classified as an ergosterol biosynthesis-inhibiting fungicide with

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protective and therapeutic properties [13]. It provides enhanced user safety and environmental protection [14].Unfortunately, the intensive and excessive use of these pesticides leads to residues in food commodities and the soil ecosystem. The study of the contamination of various components of the environment through the persistence and dissipation of pesticide residues should be estimated in plants and soil systems [15-16]. Consequently, the pesticide residue determination in food is of great importance as a major food safety concern as some of these pesticides exceed MRL values when not used in accordance with GAP [17-20].

Thus, the present study aimed to assess human health risk, and study the persistence and dissipation of two fungicides, Flutianil and Propiconazole (comparing the already used propiconazole as reference for the recently used flutianil) used on green beans (leaves and pods) by the quick, easy, cheap, effective, rugged, and safe (QuEChERS) method followed by residues determination using a high performance liquid chromatograph (DAD-HPLC).The work published using these two pesticides on green beans is very few.

2. Materials and Methods

2.1. Chemicals and reagents

The pesticide standards (Flutianil and Propiconazole) (Fig.1) Structure of spirodiclofen and propiconazole were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany) with 93 and 98.50% purity, respectively. The formulations (Gatten 5% EC and Tilt 25% EC) were obtained from the Central Agricultural Pesticide Laboratory (CAPL), Agricultural Research Centre (ARC), Giza, Egypt. All solvents were of HP grade. The QuEChERS salts MgSO4, NaCl, trisodium citrate dihydrate, disodium hydrogen citrate sesquihydrate, and d-SPE salts were purchased from Agilent Technologies (Wilmington, DE, USA). Micropore filters of 0.2 m were purchased from Whatman (USA).

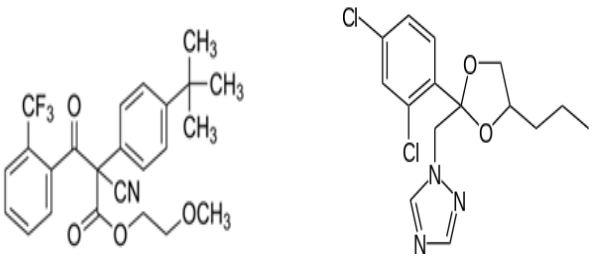

Fig.1 Structure of spirodiclofen and propiconazole

Standard preparation

Stock solutions were prepared at a concentration of 100 mg/ml separately in acetonitrile and stored at 0–5°C. Calibration standards and working solutions in concentrations ranging from 0.01 to 10 ppm mg/L were prepared by serial dilution of the stock solutions.

2.2. Field experiments

The experiment was conducted according to the recommended agronomic practises for cultivation in February 2022 in the faculty of Agriculture at Cairo University. Green bean (*Phaseolus vulgaris* L.) variety Hama was grown in a green house in double rows 1.0 m wide and 0.5 m apart in the row and grown in an area of 175 m^2. The experimental area was divided into four plots with a randomized complete block design with three replicates, beside control plots which were sprayed with water. After about 70 days of cultivation, plants were sprayed with commercial formulations of [Flutianil (Gatten 5% EC 20 cm^3, and Propiconazole (Tilt 25% EC 15 cm^3)] /100L water. A knapsack sprayer was used to spray at the recommended dosages.

2.3. Sampling and storage

After pesticide application, random sampling of green bean pods and leaves (1 kg) was performed from control and treated plots at 0 (2 h), 1, 3, 5, 7, 10, 15 and 21 days after the application to study the dissipation of the pesticides according to the FAO/WHO guidelines [21]. All samples were transported in labeled polyethylene bags in darkness to the laboratory.

2.4. Extraction and clean up

All leaves and pods samples were homogenised using a Hobart Food Chopper (Model: 84181D, OH, USA) and all samples were stored in a deep freezer at –18°C until further procedures. Samples were prepared using a modified QuEChERS method according to [22]. For leaves and pods, a 10 g sample was weighed into a 50 mL centrifuge tube and 2.5 mL of distilled water was added. The tube was shaken well for 1 minute by hand. Extraction was held by adding 10 ml of acetonitrile ACN to all samples, vortexed for 1 min. To get rid of water and induce separation, salts were added; 4g magnesium sulphate, 1g sodium chloride, 1g sodium citrate

dehydrate, 0.5g sodium citrate sesquihydrate, shaken well and centrifuged (Centrifuge model: Beckman J2-MC) at 3400rpm for 5 minutes. Supernatant (1 ml) was filtrated through a 0.22m filter and kept in glass vials until determination.

2.5. Instrument conditions

An aliquot of the extract was injected into the Agilent HPLC 1100 (Agilent, Palo-Alto, CA, USA), equipped with a diode array detector (DAD) and a dual pump. Separation was conducted using an Agilent reversed phase ZORBAX Eclipse Plus C18 column (250x4.6mm id and 5 m particle size), through a 20-ul loop. The column temperature was 25°C with a flow rate of 1 mL/min. The mobile phase, detection wavelength, and Rt of each pesticide are mentioned in Table 1.

2.7. Human health risk assessment conditions

The increasing global concern with the risk of intensive and extensive use of pesticides to secure food has made it a priority to assess the risk of pesticide use on health. Due to the different and diverse uses of beans, this assessment was a must. Risk was assessed by calculating dietary exposure and Maximum Permissible Intake (MPI) as confirmation for both pesticides under test, according to the adult mean body weight (60 kg), and the acceptable daily intake (ADI) risk measurement was calculated

MPI=ADI x average body weight (60kg)

2.8. Method validation

Fortified samples were prepared by adding different standard solution concentrations to 10 g of control samples of pods and leaves, resulting in the levels of (0.01, 0.1, 1, 5, and 10 mg/kg). The fortified samples were left for 30 min. standing at room temperature to allow suitable penetration of the pesticide into the matrix before extraction. Each fortification level was analysed through six replicates, which passed through the whole process of extraction, clean-up, and analysis as described above. Matrix Effect: It was calculated using the following equation [23].

$$\textbf{Matrix effect (\%ME)} = (S1/S2 \times 100) - 100 \quad (1)$$

S1: the slope of standard curves of sample matrix
S2: the slope of standard curves of pure solvent.

ME% > 0 represents enhancement, ME% < 0 represents suppression and ME% = 0 indicates no matrix effect.

The methods were evaluated according to different validation parameters, including limit of detection (LOD), limit of quantification (LOQ), linearity, and accuracy and precision. The standard calibration curves were obtained by plotting the peak area against the concentration of the corresponding calibration standards at five calibration levels ranging between 0.01 and 10 mg/kg.

LOD is known as 3σ/S and LOQ is defined as 10 σ /S. Where σ is the standard deviation and S is the slope of the calibration curve.

The linearity of the method was tested to exhibit a relative relationship between the pesticide concentration in the working range and the detector response to it [24]. Precision in the case of repeatability (RSD) was performed at the same fortification levels by including six replicates on the same day.

3. Results and Discussion

3.1. Method Efficiency

Validation study: The method was evaluated by studying different parameters, including linearity, limit of detection (LOD), limit of quantitation (LOQ), accuracy and precision [25]. Flutianil, Propiconazole treated samples were extracted and cleaned up using QuEChERS method. The samples obtained were analyzed using HPLC equipped with a diode array detector (DAD). A 20ul volume was injected. The used pesticides were identified by comparing their retention times (Rts) with that of the reference standard using the same solvent system in HPLC (Table 1). The LOD and LOQ values were found to be 0.01 and 0.05 mg kg -1 respectively.

Table 1. Chromatographic analysis conditions and statistical parameters of Flutianil, Propiconazole and by HPLC-DAD with ZORBAX Eclipse plus C18 column.

Pesticide	Mobile phase	Rt (min.)	Wave length (nm)	LOD (mg/ kg)	LOQ (mg/ kg)
Flutianil	acetonitrile: water (90:10, v/v)	2.99 +0.01	210	0.059	0.017
Propiconazole	acetonitrile: Methanol (65:35, v/v)	3.12 +0.01	220	0.05	0.01

Linearity: The reliability of the method was evaluated by linearity, which was evaluated by calibration curves set for each compound by injections in triples (n=3) for five concentrations (0.01 to 10 mg kg -1 each). All tested compounds showed a good linear relationship with the regression coefficient (R^2) which was done by statistical data obtained with a correlation coefficient of 0.9956 for flutianil (Figure 2) and R^2 was 0.9999 for propiconazole (Figure 2).

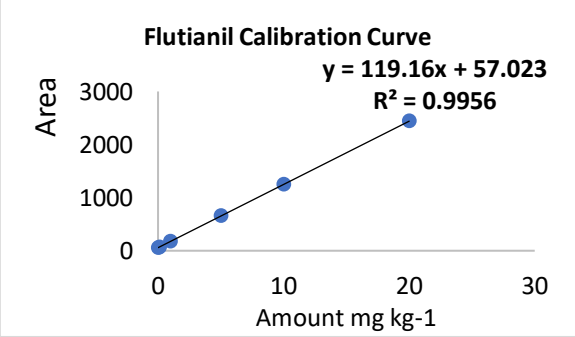

Figure 2. Calibration curve of different concentrations of flutianil standard

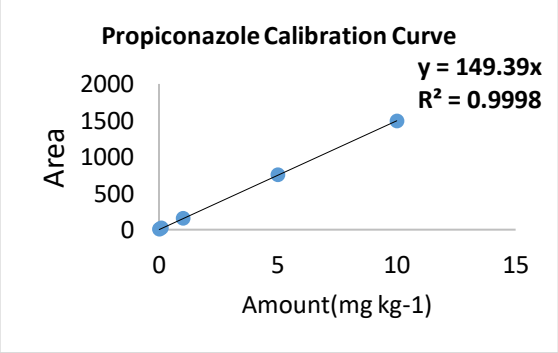

Figure 3. Calibration curve of different concentrations of propiconazole

For method accuracy evaluation, blank samples were fortified, each tested pesticide, with five levels ranged which from 0.01 to 10 mg kg-1 and six replicates each n=6 with injections. The accuracy of the method was evaluated by the calculation of the recovery average at the tested levels.

Recovery Results: The reliability and validity of the analytical method was done by fortification experiments. Control samples of green bean leaves and pods were spiked at 0.01, 0.10, 1.0, 5.0 and 10.0 mg kg−1 levels, processed as described above and residues were quantified. The recoveries of flutianil in fortified leaves ranged between 89.2 and 110.2%, in pods were from 85.0 to 99.0%. Propiconazole recoveries at the same spiking levels in leaves ranged between 76.9 and 99.2%, in pods between 91.0 and 100.0% (Table 2).

Precision was performed at the same fortification levels by six replicates on the same day and was calculated as relative standard deviation (%RSD). The precision and accuracy were considered adequate for validating the method according to the validation criteria. It was confirmed that the method adopted was considered reliable with flutianil, and propiconazole analysis and proof of accurate and precise work. Recovery ranged from 76.9% to 110.2 % for the tested pesticides. RSD% was 1.6-15.4% and 0.5-14.2 % for flutianil and propiconazole, respectively. All calculated recoveries mean results spanned from 70% to120% and RSD <20%. The MRL values were 0.01, the method used must fit for the intended purpose and provide reliable results [26-27].

Table 2. Recovery of flutianil and propiconazole spiked in green bean leaves and pods samples

Fortified Level mg/ kg	Flutianil				Propiconazole			
	Pods		Leaves		Pods		Leaves	
	Rec. % (n=6)	RSD %	Rec. % (n=6)	RSD %	Rec.% (n=6)	RSD %	Rec. % (n=6)	RSD %
10	99.0	2.4	110.2	1.6	99.8	1.0	99.2	0.5
5	97.5	2.8	93.9	6.5	93.5	3.8	95.0	5.3
1	92.7	2.5	89.2	15.4	92.7	3.8	86.5	4.8
0.1	85.0	7.5	93.8	6.2	91.0	5.5	76.9	14.2
0.01	95.9	7.1	94.8	7.6	100.0	5.4	88.3	7.5

Matrix effect

Table 3: Matrix effect in the different matrices

Matrix	Propiconazole	Flutianil
Pods	-8.77	23.41
Leaves	-6.32	-3.06

Matrix effect ranged from -13.38(Suppression) to 23.41, for each pesticide and matrix under test. ME% > 0 represents enhancement, ME% < 0 represents suppression and ME% = 0 indicates no matrix effect [23].

3.2. Persistence and dissipation of flutianil, propiconazole and in green bean leaves and pods

The data of the dissipation of flutianil in green bean leaves and pods are presented in Table 4 and Fig. 4. The initial deposits of flutianil in leaves and pods were calculated to be 4.51 and 3.06 mg kg−1 for the recommended dose, respectively. One day after application, the residues dissipated by 42.35% in leaves and 51.3 % in pods.

However, the residues after day 3 were 0.827 mg kg−1 with a percent dissipation of 81.66 in leaves, while in pods the residues were 0.52 mg kg−1 which dissipated by 83.01 %. The residues in leaves were below the quantification limit (0.05 mg /kg1) on the 5th day. On the 7th day, the dissipation rate in pods was 97.8%, while in leaves no residues were detected.

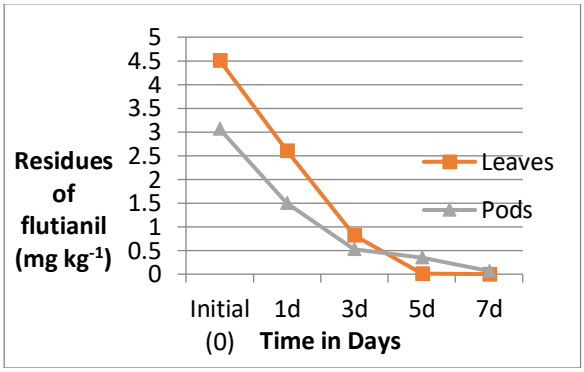

Figure 4. Dissipation of flutianil residues (mg/ kg) in green bean (*P. vulgaris* L.)

Table (4): Residues of flutianil in leaves (L) and Pods (P) at different time intervals

Time after application in days	Residues of Flutianil (mg kg−1)					
	Leaves			Pods		
	Initial deposit	Dissipation %-L	RSD	Initial deposit	Dissipation %-P	RSD
Initial (0d)	4.51 ±0.4	0.00	8.38	3.06 ±0.04	0.00	1.52
1d	2.6 ±0.3	42.35	10.69	1.49 ±0.1	51.30	8.91
3d	0.827 ±0.2	81.66	6.53	0.52 ±0.06	83.01	11.61
5d	0.0097 ±0.01	99.78	10.59	0.35 ±0.02	88.56	9.37
7d	Nd	---	--	0.065 ±0.03	97.87	6.38
10d				Nd		
PHI(days)	10					
MRL	0.01					

The initial residues were higher on leaves than on pods, which may be due to the shape of the leaf compared to that of a pod. The values of initial deposit of Propiconazole on leaves and pods were recorded at 5.65 and 2.48 mg/ kg, respectively. After one day of pesticide application, the residues dissipated by 59.82 in leaves, which was followed by a gradual decrease 69.73, 83.24 and 99.85 % after 3, 5 and 7 days of application, no residue was detected after 10 days. In pods, the behavior was different. The percent of dissipation was 33.87%, which increased highly to reach 60.8% after 3 days and 7 days after application it reached 90.32%, also with no residues detected after 10 days. The residues in leaves were below the quantification limit (0.05 mg /kg) on the 7th day (Table 5and fig. 5).

Table (5): Dissipation of Propiconazole in leaves (L) and Pods (P) at different time intervals

Time after application in days	Residues of Propiconazole (mg kg−1)					
	Leaves			Pods		
	Residues detected (mg kg−1)	Dissipation %-L	RSD%	Residues detected (mg kg−1)	Dissipation %-P	RSD%
Initial (0d)	5.65 ±0.58	0.00	10.18	2.48 ±0.28	0.00	11.42
1d	2.27 ±0.19	59.82	8.58	1.64 ±0.12	33.87	7.9
3d	1.71 ±0.05	69.73	2.84	0.99 ±0.05	60.08	1.02
5d	0.947 ±0.05	83.24	5.92	0.961 ±0.07	61.25	7.77
7d	0.0082 ±0.02	99.85	7.87	0.24 ±0.03	90.32	16.46
10d	Nd			Nd		
PHI(days)	10					
MRL	0.01					

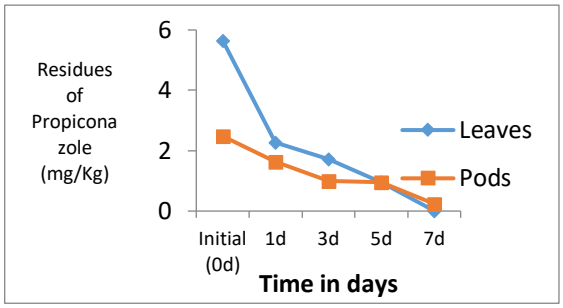

Figure 5. Dissipation of propiconazole residues (mg/ kg) in green bean (*P. vulgaris* L.)

The rate of dissipation of flutianil was faster and higher than propiconazole, which may be due to the percent of active ingredient, which was 5 EC% for flutianil, while propiconazole was 25EC %.
The results agree with those of [28] who examined, Flutianil residues in agricultural commodities (pepper, sweet pepper, mandarin, hulled rice, soybean, and potato) spiked with 0.02 or 0.2 mg/kg flutianil. The average recovery of flutianil was 76.5-108.0% with a relative standard deviation of less than 10%. The limit of detection and limit of quantification were 0.004 and 0.02 mg/kg, respectively. The results obtained by [13] showed that the propiconazole recoveries in leaves ranged from 79.8 to 92.1% in banana leaves, 84.6-92.4% in fruits. The linearities for all analytes were R2 ≥ 0.9953 with a recovery range of (74.5–106.4%). The limit of quantification (LOQs) for the tested analytes was 10 µg kg−1.The result of recoveries and relative standard deviation were in line with [29].As for Propiconazole, the solubility in water is moderate, 100 mg/l at 20 °C, the log octanol-to-water partition coefficient (log Kow) is 3.72 at neutral pH, it is only very slightly volatile, and systemic All these properties lead to dissipation of 33.87, 60.08, and 61.25% after 1, 3 and 5 days after application [30].
Meanwhile, Flutianil's (log Kow) value is 6.5and the solubility in water is very low, 0.1 mg/l at 20 °C, it is also fat soluble and translaminar. Its dissipation was 51.30, 83.01,and 88.56% after 1, 3 and 5 days of application .The water content in the pods is about 50%.
All these different factors combined together affect and control the dissipation of pesticides in addition to solubility, systemicity, physical and chemical factors such as volatilization, photochemical degradation; chemical and biological transformation, leaching and sorption, light, heat, pH, moisture and growth dilution factor, controlled the dissipation rate and behavior of both tested pesticides [31]; [32]; [33] and, [34].

Table (6): Maximum Permissible intake and Dietary Exposure for Flutianil and Propiconazole in Green Beans Edible Part (Pods)

Days (after application)	Flutianil in Green Beans Pods			Propiconazole in Green Beans Pods		
	Maximum Permissible Intake (MPI)mg person−1 day−1	Residues mg kg	Dietary exposure mg person−1 day−1	Maximum Permissible Intake (MPI)mg person−1 day−1	Residues mg kg	Dietary exposure mg person−1 day−1
0	49.2	3.06	0.0395	2.4	2.48	0.0320
1	49.2	1.49	0.0192	2.4	1.64	0.0212
3	49.2	0.52	0.0067	2.4	0.99	0.0128
5	49.2	0.35	0.0045	2.4	0.961	0.0124
7	49.2	0.065	0.0008	2.4	0.24	0.0031
10	49.2	ND	ND	2.4	ND	ND

3.3. Risk Assessment

In this work, risk is assessed by calculating dietary exposure and Maximum Permissible Intake (MPI) as confirmation for both pesticides under test, according to the adult mean body weight (60 kg) and the acceptable daily intake, risk measurement are calculated. Dietary exposure is less than the maximum permissible intake, which appears to be human safe [35],) [36].

Food safety of Flutianil and propiconazole was conducted by calculating dietary exposure justified by maximum permissible intake (MPI).The ADI of flutianil was 0.82 mg kg^{-1} b.w, [37] and of Propiconazole was 0.042mg/kg bw/day [38] .

Considering the mean body weight of an adult was 60 kg, MPI was calculated by multiplying the ADI by 60 kg, resulting in 49.2 and 2.4 mg person^{-1} day^{-1}. EFSA concluded that the short-term and long-term intake of residues resulting from the use of flutianil according to the reported agricultural practices, is unlikely to present a risk to consumer health, [39].

4. Conclusions

The residues and safety to humans of flutianil and propiconazole in green beans under greenhouse conditions was evaluated. Extraction was performed applying (QuEChERS) followed by HPLC-DAD for quantitative estimation of the residues. The used method was fit for purpose. The tested compounds showed good linearity with regression coefficient (R^2) of 0.9956 and 0.9999, with high accuracy, precision, matrix effect, satisfactory accepted recoveries (76.9%–110.2%) and relative standard deviation (<20%). The limits of detection and quantification were 0.01 and 0.05 µg/kg, respectively. Pre-harvest intervals (PHIs) were 10 days and maximum residue limits (EU MRLs) was 0.01 for both pesticides. The health risk assessment showed that green beans treated with both pesticides are safe to human.

5. Conflicts of interest

There are no conflicts to declare.

6. Acknowledgments

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