Dissipation of spiroxamine residues in open field cucumber and dietary risk assessment

F. Malhat^{1,*}, Ch. Anagnostopoulos^{2,*}, S. El-Sayed¹ and S. Abdelsalam Shokr¹

Summary Spiroxamine is one of the most used fungicides in the Mediterranean region, in significant uses such as open field cucumber. Residue trials in the Northern part of Egypt were conducted to investigate the dissipation of spiroxamine (SPX) in cucumbers according to the authorized Good Agricultural Practice (GAP) (1 x 75 g a.i. ha⁻¹ at BBCH 85 to 89) and more critical use patterns. SPX was extracted from cucumbers using a modified QuEChERS protocol and residues were analyzed by liquid chromatography with tandem mass spectrometry (LC–MS/MS). The method was successfully validated with an LOQ of 0.001 mg kg⁻¹. A steep decline of SPX residues in cucumbers fit a first-order decay process with a calculated $t_{1/2}$ of approximately 2 days and almost complete degradation (99%) after 14 days. Chronic and acute exposure calculations were performed for cucumbers treated with SPX according to all tested GAPs employing two different approaches. In all cases a health risk after consumption of cucumbers was not identified.

Additional keywords: consumer exposure, field trial, kinetic model, LC-MS-MS, pre-harvest interval, spiroxamine

Introduction

Cucumber (*Cucumis sativus* L., Cucurbitaceae) is a popular vegetable, and a significant part of the Mediterranean diet, being a source of vitamins and minerals, and is consumed mostly raw. The cultivation of the vegetable in warm climates is susceptible to various disease infestation, especially under open field conditions, with fungi diseases such as *Fusarium oxysporum* f.sp. *cucumerinum*, *Alternaria alternata*, *Verticillium* sp., *Botrytis cinerea*, *Pseudoperonospora cubensis*, *Erysiphe cichoracearum*, *Sphaerotheca fuliginea* and *Leveillula taurica* being some of the most serious problems, effecting both yield and quality of the final product.

To protect cucumber cultivation in Egypt, several fungicides (active substances)

are authorized by the Egyptian Agricultural Pesticide Committee (APC) with spiroxamine (SPX) being one of them. SPX is authorized in many countries for various uses such as *Uncinula necator* in grapes (Hellenic Ministry of Rural Development and Food), bananas (European Food Safety Authority, 2015) and cereals in combination with triazole fungicides, like prothioconazole (European Food Safety Authority, 2010). Although, these authorized uses are supported by residue data provided by the industry, in open literature, research studies investigating the residue behavior of SPX in cucurbits are not available. However, studies are available on the investigation of the residue behavior in grapes (Tsiropoulos, 2005a; Tsiropoulos, 2005b), strawberries (Malhat, 2020) or on the metabolism in various crops (Buerge *et al.*, 2016) and soil (Rosales-Conrado, 2009; Sukul *et al.*, 2010; Baćmaga *et al.*, 2019). In addition, several studies are available assessing parameters related to the efficacy of the active substance, in general (Miller and Gubler, 2004) or in specific crops like citrus (D'Aquino *et al.*, 2011).

Residues of SPX are detected in various food commodities of plant origin like bananas, peppers, grapes, wheat and melons

¹ Pesticide Residues and Environmental Pollution Department, Central Agricultural Pesticide Laboratory, Agricultural Research Center, Dokki, Giza 12618, Egypt.

² Benaki Phytopathological Institute, Scientific Directorate of Pesticides Control and Phytopharmacy, Laboratory of Pesticides Residues, 8 St. Delta Street, GR-145 61 Kifissia, Attica, Greece.

* Corresponding authors: farag_malhat@yahoo.com
c.anagnostopoulos@bpi.gr

(European Food Safety Authority, 2020), animal origin like eggs (Pereira, 2014) and environment samples like pollen of field bean, strawberry and raspberry (Tosi, 2018; David, 2020), and water (Tokatli, 2020). Exposure to this compound might have detrimental health effects on humans, thus dietary toxicological reference values are set. An acceptable daily intake (ADI) of 0.025 mg/kg bw/d by applying an assessment factor of 100 to the no-observed-adverse-effect level (NOAEL) from the 1-year dog study and an acute reference dose (ARfD), is 0.1 mg/kg bw derived from the acute neurotoxicity study in rat, with a safety factor of 100 are adopted at European (EU) level (European Food Safety Authority, 2010; European Commission, 2020). Therefore, it is important to minimize these residues to a reasonable level by establishing maximum residue limits (MRLs) in cucumbers. After a search by the authors in several databases (BCGlobal's Pesticide Database; Codex Pesticides Residues in Food Online Database; European Commission 2020), established MRLs on SPX in cucumber were not identified. In the EU the default value of 0.01 mg kg⁻¹ is used for enforcement (European Commission, 2016).

In this study, the dissipation kinetics of SPX in Egypt following several use patterns, was investigated. A risk assessment of the residues resulting from these practices was conducted and the safety of the maximum residues was estimated at several Pre-Harvest intervals (PHIs).

Materials and Methods

SPX was applied using a commercial EC formulation, in outdoor field trials following several GAPs at Matshtul district (Sharkia governorate, Egypt) in one growing season (2018-2019). According to regulatory guidelines (OECD, 2009) in the case of outdoor crops the results on pesticide residues can be extrapolated to other regions with the same climate, this being a common practice in the EU (European Commission, 2017; European Commission, 2013). Although the Mat-

shtul district is on the Mediterranean part of Egypt, residue studies of SPX in other Mediterranean countries, especially EU countries, were not available from the literature and hence a comparison of the results was not possible.

A randomized block design (15 m² plot size) was employed for the plot/crop layout with triplicate treatments including a buffer area with no pesticide application. An additional plot was included in the design as blank control plot. The pesticide was applied once, twice or three times at a single (75 g a.i. ha⁻¹) or double (150 g a.i. ha⁻¹) dose using a high-pressure knapsack sprayer. The first spray was applied at BBCH 85 – 89 (half of the size of fruit maturation) and with a 14 days interval for the GAPs with more applications. The authorized GAP (1x75 g a.i. ha⁻¹) and additional more critical use patterns (double dose rate or 2-3 applications or both) were investigated as to estimate the final residue and risk to the consumer from a potential misuse or emergency applications due to unexpected disease or pattern that may be authorized in the future.

For each field, fruit samples (2 kg) were randomly collected at 3 and 7 days, except from the fields in which application was conducted according to the authorized (1 x 75 g a.i. ha⁻¹) and the double dose pattern (1 x 150 g a.i. ha⁻¹). In the latter fields, samples were collected at 0, 1, 3, 7, 10 and 14 days after the final application, packed in well-aerated bags and brought to the laboratory in the same day. The storage period between harvest and analysis was less than 1 week, thus investigation of the stability of residues during storage is not required (European Commission 1995; OECD 2007). For the analysis of the samples the (Quick, Easy, Cheap, Effective, Rugged and Safe) QuEChERS extraction procedure (EN-15662 2009) as previously modified by the authors (Malhat et al. 2020) was used. Related to the materials used in the analysis, SPX certified reference standard (99%) was bought from Dr Ehrenstorfer (Augsburg, Germany), all solvents (acetonitrile and methanol) were HPLC grade and purchased from Sigma Al-

drich Chemical Co., analytical grade sodium chloride (NaCl), magnesium sulfate anhydrous (MgSO₄) (dried at 450°C overnight before use), trisodium citrate dehydrates and disodium hydrogen citrate sesquihydrate were supplied from Merck (Darmstadt, Germany). The sample preparation is described below for completeness:

Ten grams of the homogenized sub-sample was weighted into 50 mL centrifuge tube along with 10 mL of acetonitrile and vortexed for 2 min. Hereafter, 4.0 g of MgSO₄ anhydrous, 1.0 g of NaCl, 1.0 g tri-sodium citrate (dihydrate) and 0.5 g sodium hydrogencitrate sesquihydrate were added and the tube was shaken intensively for 2 min. by hand. The sample was then centrifuged at 4800 rpm for 5 min for phase separation and an aliquot of 200 µL of supernatant was diluted with 800 µL acetonitrile, filtered with a PTFE (0.2 µm) syringe filter and the samples were ready for injection in the LC-MS/MS. Based on this procedure, the C (mg kg⁻¹) in the sample correspond to 5 times the concentration in the extract (µg mL⁻¹) analyzed. An LC-MS/MS system with an Exion LC system coupled to QTRAP mass spectrometer (QTRAP 6500⁺, AB SCIEX) was used for the analysis of SPX residues. A Synergi C₁₈ column, 2.5 µm Fusion-RP 100Å, 3.0 x 50 mm (Phenomenex) was used for chromatographic separation. The column temperature was held at 40°C. Mobile phases consist of water (A) and methanol (B) both containing 5 mM ammonium formate.

Elution was performed at gradient mode as follows: a ratio of 20% for eluent B, running for (0-1.0 minutes), 20-100% B (1-8 min.), and then 20% B (8.05–10 min.). at a flow rate of 0.4 mL/min. The injection volume was 2 µL. SPX was eluted at 5.5 min. The LC-MS/MS was operated using electrospray ioniza-

tion (ESI) in the positive ion mode with multiple reaction monitoring (MRM) in the scan mode. Sources and gas parameter were optimized as follows: ion spray voltage 5500 V for ESI (+); ion source temperature 400°C; curtain gas 20 psi; collision gas medium; nebulizer gas and auxiliary gas 35 psi. The Analyst software (Version 1.7.1, AB Sciex) was used for the instrument setting, data acquisition and processing. The specific parameters are given in Table 1.

The method was validated, determining the limit of quantitation (LOQ), accuracy and precision (recoveries from fortified samples), matrix effect (due to co-extractives) and linearity (in solvent and matrix) as per SANTE guideline (Commission 2017). The matrix effect (ME) was evaluated according to the following equation:

$$\%ME = 100 \left(\frac{M_{matrix} - M_{solvent}}{M_{matrix}} \right)$$

where %ME is the % estimation of the matrix effect, M_{matrix} and $M_{solvent}$ are the slopes of calibration curves in cucumber extract and acetonitrile, respectively. If the ME value is between the threshold of ±20 %, matrix effect can be considered not significant (Kmellár *et al.*, 2008; Pizzutti *et al.*, 2009). In addition to the above threshold, according to Ferrer *et al.* (2011) matrix effect can be considered as soft when ME is calculated between 20- 50% and strong above 50 % or below -50%.

The dissipation rate of SPX in cucumber was evaluated by subjecting the data to a first-order kinetics equation:

$$C_t = C_0 e^{-kt}$$

Table 1. LC-MS/MS optimization parameters for SPX.

Precursor ion (Q1)	Product ions (Q3)	Retention time	Declustering potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision Cell Exit Potential (CXP)	Purpose
298.3	144.2	5.5	80	10	27	4	quantitation
298.3	100.2	5.5	80	10	50	4	qualifier

where C_t represents the concentration (mg kg^{-1}) of SPX at time (t), C_0 represents the initial concentration (mg/kg) of SPX after application, and k is the dissipation coefficient in day 0.

The persistence of SPX is generally expressed in terms of half-life, $t_{1/2}$ or DT_{50} , i.e., time for the disappearance of pesticide to 50 % of its initial concentration and was calculated from the k value as following:

$$t_{1/2} = \ln 2 / k$$

To ensure that a health risk is not related to the current use, a dietary consumer risk assessment was performed, including as input values for the exposure the residue levels from all PHIs related to the authorized GAP; for the hazard the ADI (0.025 mg/kg bw/d) and ARfD (0.1 mg/kg bw/d) was taken into consideration (European Commission 2020; European Food Safety Authority, 2010).

The long-term dietary exposure to SPX from cucumbers consumption was estimated using the following equations (Wang *et al.*, 2015; Zhu Xiaodan *et al.*, 2016) (FAO, 2009; FAO/WHO, 1988):

$$\text{NEDI} = \text{CRL} \times \frac{F}{60}$$

where NEDI is national estimated daily intake (mg/kg, bw), CRL is residue levels (mg/kg); F is the cucumber average consumption data (g/d) according to WHO cluster diet G06 (IEDI 2014; WHO 2013a; WHO 2013b) and 60 is the body weight (kg).

and

$$\text{RQ} = \frac{\text{NEDI}}{\text{ADI}}$$

where RQ is the risk quotient and ADI is the acceptable daily intake (mg/kg, bw). Whereas RQ value higher than 1 ($\geq 100\%$ of ADI) an unacceptable chronic risk to the consumer is identified.

Additionally to the above approach, since EU is a significant market for Egyptian cucumbers, the deterministic model EFSA

PRIMO revision 3.1 (European Food Safety Authority, 2019) which is the calculation model used on EUMRLs setting, was also employed.

Results and Discussion

The applied method is considered suitable for the determination of SPX in cucumbers at an LOQ of 0.001 mg kg^{-1} according to EU standards (SANTE/11813/2017, 2017). The LOQ is 10 times below the default EUMRL of 0.01 mg kg^{-1} and is suitable for investigation of the degradation of the compound at very low levels. The mean recovery values were between 99 and 105%. For the precision, both repeatability and reproducibility were estimated and were found acceptable, with RSD_r of below 17% for repeatability (at all fortification levels) and RSD_R of 11% (calculated at 0.1 mg kg^{-1}) for "day to day" reproducibility. Good linearity using 5 levels (0.001 , 0.01 , 0.02 , 0.05 , and 0.1 mg L^{-1}) was obtained in both solvent and matrix with the coefficients of determination (R^2) higher than 0.99. Matrix effect was found to be negligible (ME below 20%). Recovery and precision data are presented in Tables 2 and 3. A visual comparison of the chromatograms in blank cucumber and spiked sample at 0.001 mg kg^{-1} (validated LOQ) is presented in Fig. 1.

The dissipation rate of SPX in cucumbers was calculated based on the experiment performed with the authorized GAP from residues measured at the day of the last application (0 day), and 1, 3, 7, 10 and 14 days later. Residues were not detected in the control samples. In the samples col-

Table 2. Recovery percentage and relative standard deviation of SPX in cucumber ($n=6$).

Spiking level (mg/kg^{-1})	Recovery (%)	RSD (%)
0.001	98	17
0.01	96	15
0.1	97	10

Table 3. Recovery RSD_r and RSD_R values obtained from analysis of cucumber samples spiked with SPX at 0.1 mg kg^{-1} ($n=6$).

Analysis day	Recovery %	RSD_r %	RSD_R %
1	95	11	11
2	99	6	
3	94	3	

lected at the day of the last application (day 0) residues were 0.67 mg kg^{-1} . In the 1st day a degradation of 13% was observed with a rapidly and gradually decrease of the residues until day 7, with residue levels declining down to 0.041 mg kg^{-1} (decline of 94% compared to the initial concentration). After this point and with a stable but less steep degradation, the residue levels dropped to

A. Blank cucumber matrix

B. Spiked cucumber sample 0.001 mg L^{-1}

Figure 1. LC-MS/MS chromatograms of SPX (A) Cucumber blank (B) Spiked cucumber sample (0.01 mg L^{-1}).

0.003 mg kg⁻¹, representing almost complete degradation (>99%) at 14 days. Based on this behavior the half-life ($t_{1/2}$) was calculated at approximately 2 days (1.95) with a good exponential correlation between concentration and time ($r^2 > 0.9953$) to be observed. A graphical visualization of the dissipation pattern is presented in Fig. 2. The published work on the application of SPX in crops is scarce. In the few studies available under Mediterranean conditions, its half-life is 7 days in grapes (Tsiropoulos, 2005a) and 4.71 days in strawberries (Malhat, 2020).

The effect of the different rates and applications was also examined by comparison of the residue levels at PHI of 3 and 7 days from different use patterns. The results are

Figure 2. Dissipation pattern of SPX in/on cucumbers at the authorized GAP.

presented in tabular form in Table 4. In some cases, despite the increase of dose or number of applications, the residue levels were significantly lower compared to the less critical cases, whereas in principle an increase should have been observed. Since from the experimental conditions no error was observed or an extreme environmental parameter was not recorded, this is considered as a variability of the data set as a whole, therefore not rejected. At a PHI of 3 days, residue levels were 0.23 ± 0.005 mg kg⁻¹ when applied at a use pattern of 2×75 g a.i. ha⁻¹ and 0.43 ± 0.026 mg kg⁻¹ at twice the dose rate, indicating 47% increase in residues due to dose increase. Similar in the case with 3 applications, in which the increase was higher (68-75%). A correlation between the number of applications and the residue levels cannot be identified. In both levels in the use patterns with 3 applications, the residue levels at 3 days were lower or similar compared to the ones with 2 applications. Based on the above, the number of applications seem not to have a significant effect on the final residue. This can be confirmed by the results from both sampling days (3 and 7 days PHI).

Due to the absence of established MRL for SPX in cucumbers, a comparison is only

Table 4. Residues and % comparison of SPX in cucumbers at 2 different dosage and number of applications, more critical than the authorized GAP (1×75 g a.i. ha⁻¹).

Dosage (g a.i./ha)	n ^a	PHI	Residues (mg/kg $\pm$ SD)	Residue decline from previous sample point ^b	Residue increase due to dose rate ^c	Residue increase due to number of applications ^d
75	2	3	0.23 ± 0.005			
		7	0.012 ± 0.001	95%		
	3	3	0.11 ± 0.009			-116% ^e
		7	0.014 ± 0.011	87%		14%
150	2	3	0.43 ± 0.026		47%	
		7	0.010 ± 0.002	98%	-20% ^f	
	3	3	0.42 ± 0.032		75%	-4% ^e
		7	0.044 ± 0.001	89%	68%	77%

^a Number of applications.

^b The decline was calculated as the difference of the residues in comparison to the previous PHI.

^c Increase was calculated based on the comparison with the previous pattern with application of 150 g a.i./ha.

^d Increase was calculated based on the comparison with the previous pattern with 2 applications.

^e In these cases, residues at the GAP with 3 applications were lower than the ones in the pattern with 2 applications despite the addition residue burden due to an additional application.

^f In these cases, residues at the pattern with double the dose rate were lower than the ones in the pattern with the recommended dose despite the addition residue burden due to high dose.

Table 5. Exposure calculations based on the FAO/WHO and EFSA approach, following the authorized GAP (1 x 75 g a.i/ha).

PHI (days)	C (mg kg ⁻¹)	FAO/WHO		EFSA PRIMo rev 3.1	
		NEDI ^a (mg/kg bw/d)	RQ	Highest % of ADI	Highest % of ARfD
0	0.67	3.89 x10 ⁻⁴	0.02	4.0	44
1	0.58	3.38 x10 ⁻⁴	0.01	4.0	38
3	0.20	1.18 x10 ⁻⁴	<0.01	1.0	13
7	0.041	2.39 x10 ⁻⁵	<0.01	0.3	3
10	0.018	1.05 x10 ⁻⁵	<0.01	0.1	1
14	0.003	1.75 x10 ⁻⁵	<0.01	<0.1	0.2

^a consumption of cucumbers (34,92 gr/bw/day or 0.0349 kgr/bw/day was used.

feasible with the default value of 0.01 mg kg⁻¹. Applying the authorized GAP and based on the sampling points, residue levels in cucumbers can be considered compliant with this value after 14 days and from the dissipation pattern, after 11 days residues can be expected to be at 0.01 mg kg⁻¹ or lower.

Results of dietary consumer risk assessment are presented in Table 5. In all cases, NEDI values were far below the ADI with RQ at or below 0.02. By applying the EFSA PRIMo, the estimated chronic exposure was calculated up to 4% of the ADI. Using the same model, short term exposure was estimated at 44% of the ARfD. Based on the above, a chronic and acute risk is not identified. However, it should be noted that the above conclusion is derived from values based on a single independent field trial and although no shortcomings were observed further independent trials would be desirable to have a more robust estimation (Malhat et al., 2020).

In conclusion, a decline of SPX residues in cucumbers fit a first-order decay process with a calculated $t_{1/2}$ of approximately 2 days with almost a complete degradation (<99%) after 14 days and residues up to 0.003 mg kg⁻¹ when SPX is applied according to the authorized GAP. An increase of the dose level will result in a significant increase of the residue levels, however the contribution of the increase of the number of applications in the residues is uncertain. From the available data, cucumbers treated according to the authorized or higher dose present up

to 4% of the ADI and 44% of the ARfD using FAO/WHO and EFSA models.

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Μελέτη διασποράς υπολειμμάτων σπιροξαμίνης σε υπαίθρια καλλιέργεια αγγουριού και εκτίμηση διατροφικής επικινδυνότητας

F. Malhat, Ch. Anagnostopoulos, S. El-Sayed και S. Abdelsalam Shokr

Περίληψη Η σπιροξαμίνη είναι ένα από τα πλέον ευρέως χρησιμοποιούμενα μυκητοκτόνα στη Μεσόγειο με χρήση σε σημαντικές καλλιέργειες όπως του αγγουριού. Σκοπός της παρούσας μελέτης είναι η διερεύνηση της υπολειμματικής συμπεριφοράς της σπιροξαμίνης στην καλλιέργεια του υπαίθριου αγγουριού σε ξηρά κλίματα. Η ουσία εφαρμόστηκε σύμφωνα με την εγκεκριμένη ορθή γεωργική πρακτική και με πιο κρίσιμες πρακτικές σε υπαίθριο αγγούρι στο βόρειο μέρος της Αιγύπτου. Ο προσδιορισμός των υπολειμμάτων έγινε με την μέθοδο QuEChERS σε συνδυασμό με υγρή χρωματογραφία συζευγμένη με σύστημα διαδοχικής φασματομετρίας μάζας LC-MS/MS. Το όριο ποσοτικοποίησης LOQ της μεθόδου υπολογίστηκε στα 0.001 mg kg⁻¹. Ο χρόνος ημιζωής των υπολειμμάτων υπολογίστηκε στις 2 ημέρες με αποδόμηση περίπου 99% μετά από 14 ημέρες. Πραγματοποιήθηκαν υπολογισμοί χρόνιας και οξείας διατροφικής έκθεσης και σε όλες τις περιπτώσεις δεν παρατηρήθηκε κίνδυνος για τον καταναλωτή.

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