Dissipation and residue determination of fluopyram and its metabolites in greenhouse crops

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Short running title: Dissipation of fluopyram and metabolites in greenhouse crops

ABSTRACT

Background: Fluopyram is a pesticide widely used in tomato and cucumber crops cultivation to control fungal diseases that develop especially in environments with moderate temperatures and high humidity, such as in a greenhouse. The pathway of fluopyram dissipation has been monitored in cucumber and cherry tomato under greenhouse conditions.

Results: In the greenhouse trials, cherry tomato and cucumber were treated by irrigation water with the commercial product at the manufacturer's recommended dose and double dose. High-resolution mass spectrometry (HRMS) coupled to ultra-high performance liquid chromatography (UHPLC) has been selected as technique to obtain the identification of fluopyram and metabolites. The fate of fluopyram in greenhouse tomato and cucumber was investigated over 44 days. The metabolic pathway of fluopyram was: in a first step there was a primary transformation to fluopyram-7-hydroxy and fluopyram-8-hydroxy, isomeric compounds, and in a second phase to fluopyram-benzamide and fluopyram-pyridyl-carboxylic acid. The behavior of fluopyram does not fit any type of kinetic classical model of degradation.

Conclusions: Greenhouse trials revealed that the fluopyram is a very persistent compound, and their terminal residues do not exceed MRL at the end of the study.

Keywords: Fluopyram, Dissipation, Metabolites, Orbitrap, Greenhouse, Fungicide

INTRODUCTION

Tomato (*Solanum lycopersicum*) and cucumber (*Cucumis sativus*) are two of the most important horticultural crops in the world[1–3] and a fundamental part of the Mediterranean diet. Spain is the second Europe's greatest tomato producer and the third cucumber producer, with an average yield of $4.8x10^6$ tons/year and $643 x10^3$ tons year^{-1}, respectively. An example is the Almeria province, on the Southeast coast of Spain, which counted in 2018,[4] with 5099 and 10311 hectares of greenhouses dedicated to grow cucumber and tomato respectively, which raised a market value that exceeded one billion euros. Greenhouses are agrosystems that present important productive advantages, although their moderate temperatures and high humidity inside, and the continuous cropping practices, are favourable conditions for fungal disease development.[1] Hydroponic is often used as a soilless greenhouse cropping practice. It uses substrates such as rockwool, coconut fibre, perlite, clay pellets or vermiculite to grow the plants. This cropping technique overcomes pathogens diseases in soils and poor physical-chemical characteristics. A powdery mildew, also named Oidium (caused by *Sphaerotheca fuliginea* fungi and *Erysiphe cichoracearum* fungi), is the disease that cause major crop losses in cucumber and tomato worldwide.[2,5,6] In soilless crops, pathogens can be conserved in the substrates or in the roots that are embedded in the picks from previous crops.[7] Fluopyram[8] (N-[2-{3-chloro-5-(trifluoromethyl)pyridin-2-yl]ethyl}-2-(trifluoromethyl)benzamide) is a new systemic fungicidal compound present as the lone active ingredient in the Velum Prime® plant protection product (PPP), which is very effective against Oidium pest. It is used as a broad-spectrum fungicide applied as through drip irrigation systems or by foliar spray on several horticultural crops. Fluopyram acts on pathogen cells by inhibiting their normal respiration process. It as a systemic compound is absorbed through the leaves or roots and then distributed throughout the plant.[9–11] In consequence fluopyram can be moved easily into the edible portion of the crops which may pose a high risk to people on the basis of their

potential toxicity properties. Moreover, once the pesticide is distributed throughout the plant, it degrades into several metabolites. As a result, concern arises about the persistence of fungicide residues and its metabolites in the edible parts of crops, which could become a significant route of human exposure.[12] Usually legislation on maximum residue level (MRLs) definitions generally just includes parent compound pesticides, and only in limited cases includes certain toxicologically relevant metabolites, which result from transformations. This is the case of the MRL for fluopyram that only includes the original compound, for example, in the regulations established by Codex Alimentarius[13] or the European Union.[14] Therefore, residue of fungicides and metabolites in raw agricultural commodities must be periodically measured for food safety.[15]

This study considers, in addition to fluopyram, the characterisation of metabolites that are not included in routine monitoring programs to understand the dissipation of the fluopyram PPP, in greenhouse conditions, and to identify potential toxic metabolites that could be present in fresh tomato and cucumber vegetables.

Up to the date, fluopyram dissipation studies can be divided into those carried out in open fields and into greenhouses. Open fields studies focus predominantly on the behavior of the parent compound without taking into account the degradation compounds,[11,16–18] although in one of them a single degradation compound was identified.[9] Also there is only one study conducted in greenhouse in which its degradation compounds were studied,[19] but the crop was in soil. Concerning PPP application techniques, most of above-cited literature, deal with fluopyram foliar spraying on crops, except the study of Suchi Chawla et al.,[9] where roots were drenched in the fungicide. The main objective of this study was to deepen knowledge about the dissipation of the fluopyram pesticide, including the transformation and behavior of generated metabolites, in two important crops (cherry tomato and cucumber) under greenhouse conditions. To this end, ultra-high performance liquid chromatography (UHPLC)

coupled to high resolution mass spectrometry (HRMS), UHPLC-Orbitrap-MS, has been used in order to enhance previous methodologies based on gas chromatography with low resolution MS analyzers.[20] In addition, knowledge this is the first report in which fluopyram was applied dripping on an artificial substrate.

MATERIALS AND METHODS

Equipment, reagents and chemicals

Reference standards of fluopyram (>98.0%) and fluopyram benzamide (>97.0%) were purchased from Sigma Aldrich (St. Louis, MO, USA), while fluopyram pyridyl carboxylic acid (>95.0%) from Matrix Scientific (Columbia, USA), and were stored at −30 °C. Individual reference standard stock solutions were prepared in methanol at a concentration of 1000 mg L^{-1}. From individual reference standard stock solutions, three mix-standards were prepared by diluting accurate volumes of the above solutions to obtain concentrations of 10, 1 and 0.1 mg L^{-1} respectively in methanol. Stock and intermediate solutions were stored in amber screw-capped glass vials in the darkness at -30 °C.

The Velum-Prime® (concentrate suspension fluopyram 40% *p*/*v*) PPP was supplied by Bayer Crop Science AG. Acetonitrile and methanol (LC-MS grade) from Fluka (St. Louis, MO, USA), magnesium sulphate, primary secondary amine (PSA) and acetic acid from Panreac (Barcelona, Spain) and sodium acetate anhydrous from Sigma Aldrich were used. Filters for syringe Econofitr Nylon 0.2 µm, 13 mm were purchased from Agilent Technologies (Agilent, Santa Clara, CA, USA).

Analyses were performed with a Thermo Fisher Scientific Transcend 600 LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA), coupled to a single mass spectrometer Orbitrap Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany) that used an electrospray interface (ESI) (HESI-II). Separations were

carried out on a Zorbax plus C18 column (2.1 x 100 mm x 1.8 μm particle size) from Agilent (San Jose, CA, USA) with a flow rate of 0.2 mL min^{-1} and temperature to 25 °C. The mobile phase in gradient mode consisted of methanol as phase A, and water containing 0.1% formic acid as phase B (0-1.0 min, 5% A; 1.0-3.0 min, increased to 100% A; 3.0-7.0 min, 100% A hold for 4 min; 7.0-7.5 min 100% A decreased to 0% A; 7.5-9.0 min 0% hold for 1.5min). The total running time was 9 min. ESI parameters were as follows: spray voltage, 4 kV; sheath gas (N_2 >N95%), at 35 (adimensional); auxiliary gas (N_2 >N95%), at 10 (adimensional); skimmer voltage, 18 V; capillary voltage, 35 V; tube lens voltage, 95 V; heater temperature, 305 °C; and capillary temperature, 300 °C. Mass range used was between m/z 50–500. The Xcalibur™ software version 3.0.63, with Quan browser and Qual browser were used for optimization and quantification of the pesticide and its metabolites.[21] Data was processed with a homemade database containing the specific data of the potential fluopyram metabolites.[20] Table 1 shows the exact mass and molecular formula of the proposed metabolites.

Sample extraction

The samples were extracted following a practical method based on acetonitrile QuEChERS procedure.[22] Samples of cucumber or cherry tomato (10 g) homogenized in a 50 mL centrifuge tube containing 10 mL of 1% acetic acid in acetonitrile. Next, the tube was vortexed for 2 min. Afterward, 4 g of magnesium sulphate and 1 g sodium acetate anhydrous were added and shaken vigorously for 2 min. The tube was centrifuged for 5 min at 3061 x *g*. The supernatant was filtered with a 0.22 μm nylon syringe filter and transferred to a vial for LC analysis. Finally, ten μL were injected in the system.

Method validation

The validation study was carried out according to the performance criteria established by DG SANTE documentation[23] for fluopyram and its metabolites (fluopyram benzamide and fluopyram pyridyl carboxylic acid). Blank samples from each crop were used for validation studies. Linearity was studied in standard calibration solutions prepared in each matrix at 5, 10, 25, 50, 100 and 200 µg kg^{-1} and determination coefficients (R^2) were calculated. Trueness and intraday precision values were determined through the recovery experiments (n=5) at two spiking levels (10 and 100 µg kg^{-1}). Interday precision was estimated in the same way that intraday precision but analyzing samples in five different days. Limits of detection (LODs) were set as the minimum concentration at which the characteristic ion was monitored with a mass error lower than 5 ppm. Limits of quantification (LOQs) were estimated according to the lower concentration providing acceptable trueness and precision values.

Greenhouse trials

For dissipation trials, each crop was treated with the dose recommended (625 mL ha^{-1}) by the manufacturer and twice that dose (1250 mL ha^{-1}) by irrigation water. A second application was made in both crops, separated 30 days after the first application. Cherry tomato and cucumber were sampled at 10 min, 2 h, 6 h and 1, 2, 3, 5, 7, 9, 11, 15, 21, 28, 37 and 44 days after second application. 1 kg samples of cucumber and cherry tomato were gathered randomly from plots.[24]

The greenhouse was divided into four plots of which two were selected for the cultivation of 60 cucumber plants and two plots were selected for the cultivation of 60 cherry tomato plants, at a rate of 30 plants per plot. The experiments were conducted in a greenhouse located in Almería, on the southeast coast of Spain in 2018. Weather conditions were monitored during the study period, being the usual in this geographical region with no rain and stable temperatures, in the range of 16 - 23 ºC.

RESULTS AND DISCUSSION

Optimization of UHPLC-Orbitrap-MS

In the present study, fluopyram and its metabolites were chromatographically separated under gradient elution conditions with water (0.1% formic acid) and methanol as mobile phase components. They were analyzed with the proposed UHPLC-HRMS method (see Materials and Methods) using ESI$^+$ and ESI$^-$ modes, providing better sensitivity, good chromatographic resolution and high responses when full-scan (ESI$^+$) was applied, and then used to get the characteristic ion of the compounds that were available the standards. The product-ion spectra provided the accurate masses of fragments that were used to build a homemade database. Since primary standards are not available for some metabolites, accurate mass data from EFSA review[20] was used as a tool for mining for metabolites of fluopyram. Table 1 includes two pairs of isomers (fluopyram-7-OH and fluopyram-8-OH and fluopyram-PCA and fluopyram-PAA), although they could not differentiate between them.

Sample extraction optimization and method validation

To simultaneously measure fluopyram and its metabolites (fluopyram benzamide and fluopyram pyridyl carboxylic acid) with UHPLC-Orbitrap-MS, a QuEChERS method was applied based on the AOAC Official Method.[22]

To optimize the extraction procedure, initially acetonitrile containing 1% acetic acid (*v/v*) as extraction solvent was tested, followed by a clean-up step with magnesium sulphate and PSA. This method provided good recovery rates only for two compounds, therefore other variants were tested, such as the use of acetonitrile without cleaning step or acidified acetonitrile without cleaning stage. Best results were obtained when acetonitrile containing 1% acetic acid and without any clean-up step was applied. Table 2 shows recoveries rates obtained for

fluopyram, fluopyram benzamide and fluopyram pyridyl carboxylic acid, in both commodities at 100 μg kg^{-1}.

Once the extraction method had been optimized, the validation studies for fluopyram and its metabolites were carried out. The obtained results of the validation parameters are provided in the Table 3. The linear range was established between 5-200 μg kg^{-1}, obtaining determination coefficients (R^2) of 0.99 and residual values ≤ 20%. LODs and LOQs resulted 5 and 10 μg kg^{-1} respectively, which are below than the MRL in the EU.[14] Recovery rates at 10 and 100 μg kg^{-1} were satisfactory for fluopyram and metabolites, being between 72% and 106%, with adequate intraday, 2-15%, and interday precision, 9-17% values. The primary findings when comparing validation parameters of methods to analyse fluopyram in fruits and vegetables are summarised in Table 4. All three studies were conducted on fruits,[11,18,25] while this research concerned vegetables. Although the cited studies refer acceptable sensitivity, none of them used HRMS as detection technique. The proposed method is the first one that applies LC-HRMS and validates metabolites of the main compound.

Dissipation study in greenhouse

The dissipation process of Velum Prime® PPP was studied when it is applied in both greenhouse crops. The sampling period covers to approximately 1.5 months after the second application. Both fluopyram and metabolites were monitored and their concentrations measured in each sampling throughout the study. First application of each dose was carried out 25 days after transplanting and a second treatment was performed 30 days after of the first application in both crops. Composite samples were taken by triplicate with a size of 1 kg approximately of fruits per sample; fruits were selected using random numbers as established in the sampling regulation for the control of pesticide residues.[26] Figure 1 shows the dissipation of fluopyram in both matrices at single and double dose. It is noteworthy that the

behavior of fluopyram was similar in both target crops, and both concentrations. It is observed that the dissipation process does not conform to previous models, such as first-order kinetics, where the decrease in concentration, expressed as a percentage of the parent compound, is constant over time regardless of its initial concentration. Nor was it conform with the two-phase kinetic pattern of pesticide degradation consisting of a rapid initial decline in pesticide concentration, followed by a slower decline, as occurred in the study byChawla et al.[9] also for fluopyram. In our experiments carried out these patterns were not met, the decrease in concentration was not constant over time and varied with the initial concentration. The reason for treating with double of recommended dose was to monitor the metabolic pathway with as much of the metabolites as possible.

Residue distribution of fluopyram

Concentrations of fluopyram pesticide residues in cherry tomato dropped in the first day, after treatment at recommended dose, from 190 μg kg^{-1} obtained 10 minutes after application to 120 μg kg^{-1} 6 hours later. The same crop in the plot treated at second dose dropped from 231 μg kg^{-1} obtained in the samples collected 10 minutes after application to 92 μg kg^{-1} 2 hours later. In subsequent seven days, at normal dose, concentration increased significantly until reaches 506 μg kg^{-1}. In the plot applied at double dose, it was observed that concentration raises to the 9th day, reaching the maximum concentration of 1057 μg kg^{-1}. After that, the concentration of fluopyram pesticide residues began to decrease, the last sampling of cherry tomato, picked at 44th days after starting the study, showed the concentrations of 20 μg kg^{-1} and 97 μg kg^{-1}, normal and double dose, respectively.

Dissipation differs in the case of cucumber crop, where the initial sharp decrease of fluopyram concentration observed in cherry tomato, was not observed. On the opposite, the concentration increased in samples picked after the application until the third day, from 100

µg kg^{-1} to 615 µg kg^{-1} and from 125 µg kg^{-1} to 1033 µg kg^{-1} at normal and double dose, respectively. Then the concentrations of fluopyram residues decreased gradually to the minimum of 52 µg kg^{-1} and 119 µg kg^{-1} for both application doses respectively found in samples picked the day 44. Fluopyram pesticide remained on the cucumbers and cherry tomatoes for 44 days after the last treatment.

Moreover, the terminal residue level of the parent compound, as defined by the EU,[14] at the end of the study was below the established MRL, 900 µg kg^{-1} in tomato and 500 µg kg^{-1} in cucumber.

Residue distribution of fluopyram metabolites

Using untargeted (full-scan) acquisition mode, one more metabolite of fluopyram (fluopyram hydroxy) was detected in cherry tomato and cucumber collected from the first sampling (10 min). For further elucidation, Figure 2 shows the proposed metabolic pathway for fluopyram. Then the formation and distribution of these metabolites were studied although taking into account the impossibility of quantifying the concentration of fluopyram hydroxy, since its analytical standard was not commercially available. The way to study was through the relationship between the detected metabolite area and the fluopyram area and when representing it, concentration trend was obtained. As a result, in a first step there was a primary transformation that consisted of the hydroxylation of the parent compound to the fluopyram-7-hydroxy and fluopyram-8-hydroxy[20] metabolites, isomeric compounds. These metabolites were detected in samples from the four plots. In cherry tomato crop throughout the study a slight increase to day 7 was observed (from 0.6 µg kg^{-1} to 4.1 µg kg^{-1} in normal dose and from 0.2 µg kg^{-1} to 8.2 µg kg^{-1} in double dose), and remained at a quite constant level until the end of study (4,15 µg kg^{-1} and 8,25 µg kg^{-1} in normal and double dose respectively). Concerning both cucumber experiments, fluopyram-7-hydroxy and fluopyram-

8-hydroxy were detected in the first sample and in the following ones until the sample corresponding to 28 and 37 days, in the plots treated at normal and double dose respectively; after they were not longer detected. A second phase took place in the metabolic path: the cleavage of the hydroxylated metabolites occurs and subsequent oxidation give rise two distinct groups of metabolites: one containing the trifluoromethyl-phenyl moiety, fluopyram-benzamide (2-trifluoromethylbenzamide) and another containing the pyridyl moiety, fluopyram-pyridyl-carboxylic acid.[20] The concentrations of these metabolites showed an upward trend throughout the study: fluopyram-pyridyl-carboxylic acid concentration increased from 11 µg kg^{-1} found in the first sample of cherry tomato to 47 µg kg^{-1} detected in the last sample treated at normal dose; and from 15 µg kg^{-1} to 189 µg kg^{-1} respectively in the case of double dose treatment. In the case of cucumber, concentrations were within 4 µg kg^{-1} to 74 µg kg^{-1} (normal dose) and 11 µg kg^{-1} to 136 µg kg^{-1} (double dose). A similar behavior was observed for fluopyram-benzamide, its concentration in samples of cherry tomato varied from 7 to 26 µg kg^{-1} and from 9 to 95 µg kg^{-1} in normal and double dose, respectively. However, in cucumber samples concentrations of this metabolite ranged from 3 to 90 µg kg^{-1} (normal dose) and from 7 to 161 µg kg^{-1} (double dose), although it was undetectable on day 44.

From the quantitative and estimated information of the compounds it can be speculated that the reaction from the first transformation stage to the second one took place very quickly, since the concentration of fluopyram-hydroxy isomers has been practically maintained constant throughout the study. However, Figure 3 shows that the concentrations of the fluopyram-benzamide and fluopyram-pyridyl-carboxylic acid metabolites generally increased. It can be observed in Figures 1 and 3 that regardless of the crop, the days with higher concentration of fluopyram corresponded to those with a lower concentration of metabolites of the second transformation.

CONCLUSION

In the present study, an efficient and sensitive method of extraction based on QuEChERS method with UHPLC-Orbitrap-MS analysis was developed and validated to determine traces of fluopyram and its metabolites in the two vegetables crops (cherry tomato, cucumber). HRMS operating in full scan mode has been able to identify and measure fluopyram metabolite concentrations, which are undetectable at low concentrations for most of analytical techniques. The data obtained in the study has provided the characterisation of fluopyram pesticide dissipation during the crop of cherry tomato and cucumber under greenhouse conditions. The study includes the elucidation of metabolites (fluopyram hydroxy, fluopyram-benzamide and fluopyram-pyridyl-carboxylic acid), which have been detected from the first day to the end of the study, at 44 days. It can also be concluded that fluopyram exhibited a high persistence, since at the end of the study concentrations of the compound were still detected, although lower than MRL values. With the results of this study, progress is made in understanding the fate of fluopyram, its metabolites and its persistence. It would be necessary to consider new studies focusing on obtaining toxicological information on these metabolites, in order to consider its inclusion in the definition of MRL. The developed strategy of pesticide metabolite screening could be used to provide evidence on illegal practices if used in organic farming. This research may be the beginning to address the synthesis of main metabolites found as well as pathways of formation of these metabolites in other vegetables, their relative concentrations and possible toxicity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Table 1. List of proposed metabolites of fluopyram, their formulas and calculated exact masses

Compound	Chemical name	Formula	Exact mass
Fluopyram benzamide	2-(trifluoromethyl)benzamide	$C_8H_6F_3NO_3$	190.0474
Fluopyram-7-OH	N-{2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]-2-hydroxyethyl}-2-(trifluoromethyl)benzamide		
Fluopyram-8-OH	(N-{2-[3-chloro-5-(trifluoromethyl)-2-pyridinyl]-1-hydroxyethyl}-2-(trifluoromethyl)benzamide)	$C_{16}H_{11}ClF_6N_2O_2$	413.0486
Fluopyram-PCA	(3-chloro-5-(trifluoromethyl)-2-pyridinecarboxylic acid)		
Fluopyram-PAA	([3-chloro-5-(trifluoromethyl)pyridin-2-yl]acetic acid)	$C_7H_3ClF_3NO_2$	223.9731
Fluopyram-benzoic acid	2-(trifluoromethyl)benzoic acid	$C_8H_5F_3O_2$	191.0314
Fluopyram-methyl-sulfoxide	3-(methylsulfinyl)-5-(trifluoromethyl)-2-yridinecarboxylic acid	$C_8H_6SNO_3$	240.0033
2,9-bis(trifluoromethyl)-6,7-dihydropyrido[2,3-e] [2]benzazocin-8(5H)-one	2,9-bis(trifluoromethyl)-6,7-dihydropyrido[2,3-e] [2]benzazocin-8(5H)-one	$C_{16}H_{10}F_6N_2O$	361.0770

Table 2. Comparison of recovery values (at 100 μg/kg) when different extraction procedures were evaluated on cherry tomato and cucumber samples.

Extraction method	Recovery (%)					
	Fluopyram		Fluopyram benzamide		Fluopyram pyridyl carboxylic acid	
	Cherry T.	Cucumber	Cherry T.	Cucumber	Cherry T.	Cucumber
Acidified QuEChERS, with clean up	85	83	90	92	46	55
Acidified QuEChERS, without clean up	106	99	92	95	76	80
No acidified QuEChERS, without clean up	76	80	67	60	49	59

Table 3. Performance characteristics of the optimized method

Compound	Matrix	R^2	Concentration (μg/kg)	Recovery (%)	Intra-day precision (%RSD)	Inter-day precision (%RSD)	LOD (μg/kg)	LOQ (μg/kg)
Fluopyram	Tomato	0.99	10	93	8	9	5	10
			100	80	2	15		
	Cucumber	0.99	10	100	7	17	5	10
			100	94	4	15		
Fluopyram-benzamide	Tomato	0.99	10	72	8	10	5	10
			100	79	4	9		
	Cucumber	0.99	10	83	9	13	5	10
			100	91	10	11		
Fluopyram-pyridyl-carboxylic acid	Tomato	0.99	10	106	10	9	5	10
			100	91	15	11		
	Cucumber	0.99	10	78	7	9	5	10
			100	88	8	12		

Table 4. Comparison of validation parameters of developed method with published methods

for fluopyram in vegetables

Authors	Matrix	Recovery (%)	LOQ (µg/kg)	Metabolites incl.	Detection
This research	Cherry tomato Cucumber	72-106	10	Yes	UHPLC-Orbitrap- MS
Dong et al.[11]	Watermelon	> 91	10	No	GC-MS
Podbielska et al.[18]	Apples	70-120	10	No	GC-NPD
Abad-Fuentes et al.[25]	Strawberry	80-136	10	No	UPLC-MS/MS

Figure Captions

Fig. 1. Behavior curves of fluopyram for two spiked concentration levels: recommended dose (●) and twice dose (▲) at 2 crops: cherry tomato (a) and cucumber (b). (Error bars obtained for n = 3).

Fig. 2. Proposed metabolic pathway of fluopyram.

Fig. 3. Behavior curves of the metabolites in cherry tomato: (fluopyram benzamide (▲), fluopyram pyridyl carboxylic acid (■) and fluopyram hydroxy (●) at cherry tomato (a) and cucumber (b). (Error bars obtained for n = 3).

b)

Days after fluopyram application