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Persistence of Diflubenzuron on Conifer Forest Foliage in a Mediterranean-Climate Ecosystem Following Aerial Application

Esther Rodríguez^a; Zuriñe Gómez de Balugera^a; M. Carmen Sampedro^b; M. Aranzazu Goicolea^b; Ramón J. Barrio^b

^a Department of Chemical and Environmental Engineering School of Technical Engineering, University of the Basque Country, Vitoria-Gasteiz, Spain ^b Faculty of Pharmacy, Department of Analytical Chemistry, University of the Basque Country, Vitoria-Gasteiz, Spain

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PERSISTENCE OF DIFLUBENZURON ON CONIFER FOREST FOLIAGE IN A MEDITERRANEAN-CLIMATE ECOSYSTEM FOLLOWING AERIAL APPLICATION

ESTHER RODRÍGUEZ^a, ZURIÑE GÓMEZ DE BALUGERA^a, M. CARMEN
SAMPEDRO^b, M. ARANZAZU GOICOLEA^b and RAMÓN J. BARRIO^{b,*}

^aDepartment of Chemical and Environmental Engineering, School of Technical
Engineering; ^bFaculty of Pharmacy, Department of Analytical Chemistry,
University of the Basque Country, 01006 Vitoria-Gasteiz, Spain

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Dimilin 45 ODC (diflubenzuron) was applied in a Mediterranean-climate conifer forest, near Vitoria, Basque Country, Spain. Pine needles were collected at post-treatment 5-day intervals for analysis of the diflubenzuron residues. A liquid chromatography method with on-line diode-array and electrochemical detection was developed for the determination of the pesticide diflubenzuron and its main metabolites: 2,6-diflurobenzamide, 4-chlorophenylurea, 4-chloroacetanilide, 4-chloroaniline and *N*-methyl-4-chloroaniline in forestry matrices. Aerial application at 56.3 g AI ha⁻¹ resulted in deposition levels of the insecticide of 1080 ng g⁻¹. Within 40–65 days following treatment, 51–73% of the insecticide had been removed from the foliage. During this period, the concentration of diflubenzuron was higher than 295 ng g⁻¹. The only metabolite detected was 2,6-difluorobenzamide and this persisted on foliage until the first rainfalls occurred. An empirical mathematical correlation was found to express the influence of rainfall, solar radiation and temperature on the persistence of the insecticide.

Keywords: Diflubenzuron; Pesticides; Conifer forest; Liquid chromatography; Diode-array detection; Electrochemical detection

INTRODUCTION

Diflubenzuron, belongs to a group of compounds called benzoxyphenylureas acting as chitin synthesis inhibitors in immature stages of insects [1–3]. It is therefore used to control numerous forest and agricultural pests [4,5]. It is widely applied in the pest control of *Thaumetopoea pityocampa* (pine processionary caterpillar) in pine groves in rainy areas, as found in regions in the north of Spain.

The two main ways in which diflubenzuron degradation is produced in the environment are photodegradation and hydrolysis [6–10], the following compounds having been found to act as major metabolites: 2,6-diflurobenzamide (2,6-DFBA),

*Corresponding author. Fax: +34-945-013014. E-mail: qapbadir@vc.ehu.es

4-chlorophenylurea (4-CPU), 4-chloroacetanilide (4-CAA), 4-chloraniline (4-CA) and *N*-methyl-4-chloroaniline (NM-4-CA). Some of these metabolites, for example 4-CA and NM-4-CA, have been classified as mutagens [11].

Its persistence in forest ecosystems is not well known either due to its dependence on several factors, such as the applied dose and formulation, aerial application parameters, the number of applications, climatic conditions and the forest characteristics [12–15]. The fate and persistence of diflubenuron in various ecosystems have been a subject of interest for some years. Several of these studies have shown that the aqueous formulation of diflubenuron, Dimilin WP-25%, persists on foliage for many months [14,16,17], being a potential risk to a number of living creatures in the ecosystem [18]. For this reason, the general purpose of this study is to examine the persistence of the oil-based formulation Dimilin 45 ODC[®] on foliage of a conifer forest, in Mediterranean-climate ecosystems. A similar study on a forest in an Atlantic climate was carried out by our research team [19]. Thus, this study will compare the results obtained in both climatic ecosystems with the aim of establishing the main behavioural differences in the degradation and persistence of DFB.

For this, a suitable and sensitive HPLC method for the simultaneous determination of the pesticide and its major metabolites in forestry matrices was developed to carry out this study. The method is focused on the application of the diode-array detection (DAD) of all these chemicals on-line with oxidative amperometric detection (AD) to determine which of them can be oxidized at a glassy carbon electrode with the aim of using the electrochemical detection to improve the sensitivity of their determination. Samples must be previously extracted into acetonitrile and further cleaned-up by solid phase extraction (SPE) through aminopropyl cartridges.

EXPERIMENTAL

Chemicals

Dimilin 45 ODS was from Agrevo (Argos Schering, Alcácer, Valencia, Spain). Diflubenuron, (1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea), 4-CPU, 4-CA standards were certified 99.0% and purchased from Dr Ehrenstorfer's Laboratory (GMBH, Augsburg, Germany). 2,6-DFBA, NM-4-CA, and 4-CAA were certified 97% and supplied by Aldrich Chemical (Dorset, England). All the solvents were HPLC grade and tested for spectral purity: acetonitrile (Merck, Darmstad, Germany) and methanol (Fluka, Buchs, Switzerland). LC-grade water was prepared by purifying demineralized water in a Milli-Q water filtration system (Millipore, Milford, MA, USA). The supporting electrolyte of the mobile phase was prepared by mixing citric acid $5.0 \times 10^{-3} \text{ mol L}^{-1}$ and disodium hydrogenphosphate $1.0 \times 10^{-2} \text{ mol L}^{-1}$ to reach the desired pH, both chemicals (of analytical grade) were from Merck. Sep-Pack cartridges of aminopropyl (100 mg sorbent) tested for the clean up were supplied by Waters (Milford, MA, USA).

Analytical Methodology

The methodology, applied to this work, consists of an extraction of the compounds from pine needles, a clean-up stage to eliminate the interference of the matrix and

the subsequent analytical determination using an HPLC system with two on-line detectors, a diode array and an amperometric detector [20,21].

Extraction and Clean-up

A 3 g pine-needle sample was treated with 25 mL of acetonitrile, shaken in a capped centrifuge tube for 10 min and filtered by suction through a 0.22 μm membrane filter. A 2 mL sample of this extract was passed through a Sep-Pak cartridge of 100 mg of aminopropyl by applying a negative pressure of 933.3 Pa in a Vac-Elut Sample Preconcentration System (Varian). The cartridge was washed up with 5 mL of acetonitrile before use, for the activation of the sorbent. The eluate containing all the analytes was subsequently injected into the HPLC system. The recoveries of diflubenzuron and its metabolites from pine needles are greater than 92% for all of them, except for 4-chlorophenylurea which is 78–88% depending on the level of concentration, with relative standard deviations lower than 3.7%.

Protocol for the HPLC Analysis

The equipment was an isocratic HPLC system Hewlett Packard (Palo Alto, CA, USA) series 1050 pump fitted with a DAD Hewlett Packard model 1040 and a Data Station Vectra 486/33 N. This detector was connected on line to an AD Waters model 460 (Waters Assoc., Milford, MA, USA) with a 2.5 μL microcell, a glassy carbon electrode model 41215, a Ag/AgCl reference electrode and a data module Waters Model 745. The connection between two detectors was accomplished by connecting the exist of the optical cell of the DAD with the entry of the electrochemical cell of the AD with a stainless steel tube of 0.010 ID and 10.0 cm of length. The column was a Spherisorb ODS2 (150 mm \times 4.1 mm ID, 5 μm particle size) with a guard column (10 \times 4 mm) from Tracer (Tracer Anal., Barcelona, Spain). The mobile phase was acetonitrile–methanol–citrate–disodium hydrogenphosphate pH=7 buffer (50:2:48), pumped at a flow rate of 1 mL min^{-1} . The DAD detector was set at 245 and 260 nm, where the compounds show secondary maxima of absorbance, which do not interfere with the matrix. The working electrode of the amperometric detector was operated at 1350 mV. The detection limits were 2.2–25.0 ng mL^{-1} for the DAD and 0.07–1.7 ng mL^{-1} for the AD. This analytical method allows a suitable determination of diflubenzuron and its metabolites from pine needles, making it possible to carry out the present study of persistence of the insecticide in the conifer forest. Subsequently the limits of determination of all these compounds in pine needles were estimated: 5.35, 0.95, 1.65, and 12.04 ng g^{-1} for 4-CPU, 4-CA, NM-4-CA and DFB, respectively, at AD and 35.0, 2.3, 6.1, 5.2, 9.3 and 16.1 ng g^{-1} for 2,6-DFBA, 4-CPU, 4-CAA, 4-CA, NM-4-CA and DFB, respectively, at DAD.

The utilisation of the registered chromatographic information at DAD or AD detectors was only conditioned by the required detectability. If possible, the signals at DAD were used to evaluate the concentration of diflubenzuron and its metabolites and, in this case, spectral analysis was carried out to evaluate peak purity.

Study Site and Pesticide Application

This study was carried out in the Montemayor conifer forest on Arcamo Hills, situated in the southwest of the Province of Alava in the Basque Country, in the north of Spain.

The climate in the ecosystem is Mediterranean. The forest, covering 37 ha, contains *Pinus nigra laricio*, *Pinus pinaster*, *Pinus radiata* and *Pinus sylvestri*. These specimens were randomly distributed in the forest, although *Pinus pinaster* and *radiata* with a tree-height between 2 and 5 m are the more abundant species. The Mountain Service of Alava Province (Spain) carried out the aerial application of diflubenzuron on 30th of August 2000. A Piper Brave aircraft, fitted with six Micronair AU 5000 atomizer units, which supply droplet sizes of 100–125 μm , with a performance of 9 ha min^{-1} , was used for spray application from an approximate height of 30 m. A dose of 56.3 g of AI ha $^{-1}$ (125 cm 3 Dimilin in 5 L diesel oil) with a volume rate of application of 5 L ha $^{-1}$ was used.

Daily solar radiation (S , cal cm^{-2}), mean temperature (T_m , °C), rainfall (R , mm) and relative humidity (H_r , %) were obtained from the nearest meteorological observatory to Montemayor Forest, situated in the same climatic area: Observatory E049, 4 km from *Hereña*, in the municipality of Subijana-Morillas (Alava, Spain). This observatory is run by the Official Meteorology Service of the Basque Government (Spain).

Sampling of Foliage

Geographical characteristics of the ecosystem, with a slope (20–35%) and orientation (southwest) are uniform, therefore it is not necessary to divide the system in order to study the persistence of diflubenzuron. The sampling procedure was designed following the recommendations for sampling in forests [22]. The forest of 37 ha was subdivided into plots. These plots were randomly spread over the zone at a rate of 1 plot per 2 ha. The number of sampled trees was 4 per ha, which were randomly chosen inside the plots. Either the plots or the trees in the plots were suitably labelled according to their exact position. The samples were portions of branches (30–40 cm). The number of samples per tree (2–3) and their length were proportional to the foliage density of the tree. All the samples were randomly chosen from the top of the trees. Sampling frequency was 4–5 days at the beginning of the fieldwork and was later increased to 7 days.

All the samples of the same plot were bagged together, sealed, labelled and transported to the laboratory for their subsequent analysis. Once in the laboratory, pine needles were carefully taken from the portions of branches sampled in each plot and intermingled to obtain the representative foliage of the plot. Three subsamples were randomly taken from the representative foliage and analyzed twice each sampling day. The mean value of the results obtained in these analyses was used to represent the residual concentration of DFB on foliage in the forest each sampling day.

RESULTS AND DISCUSSION

Efficiency of the Aerial Application of Diflubenzuron

The yield of the aerial application of the insecticide ($Y_A = 64.2\%$) was calculated by the percentage rate:

$$Y_A = 100 \cdot \text{DFB}_d / \text{DFB}_a \quad (1)$$

where DFB_a is the theoretical dose of DFB applied onto foliage and DFB_d is the real dose of DFB deposited on it. DFB_d (1080.5 ng g^{-1}) was detected on the first sampling day.

DFB_a (1682.7 ng g⁻¹) was estimated by the following expression:

$$\text{DFB}_a = d_{\text{DFB}} A_o \cdot 10^3 / \rho_F \rho_{t-f} \quad (2)$$

where d_{DFB} is the dose of DFB (56.3 g ha⁻¹) applied to the forest, A_o is the percentage of forest area occupied by trees (35%), ρ_F the forest density (1500 tree ha⁻¹) and ρ_{t-f} the average tree-foliage density (7.8 kg of pine needles/tree).

A yield of 64.2% leads to the assumption that a considerable quantity of the applied dose is ineffective in treating the plague and may cause collateral contamination to the ecosystem. Despite this, and for economic justifications and reasons of effectiveness, the application area of the insecticide may be considered acceptable given the yield achieved (64.2%).

Persistence of Diflubenzuron

Sampling started the day after aerial application of diflubenzuron and continued for six months, until it was no longer detectable in tree-foliage. Table I shows the values of the residual concentration of DFB on foliage (C_t) obtained each sampling day. As can be observed, after applying 56.3 g ha⁻¹ of DFB onto the whole forest, the concentration of the insecticide deposited on foliage was 1080 ng g⁻¹, 24 h after application. The persistence was 29 weeks: less than 12 ng g⁻¹ (the lower limit of reliable sensitivity of the analytical procedure). The 51% of DFB deposited on foliage was removed during the first 40 days after application. During this period, the concentration of diflubenzuron was higher than 295 ng g⁻¹. Therefore, it is during this period that living beings are exposed to higher concentrations of DFB, above 530 ng g⁻¹. Within 40–65 days following treatment, 51–73% of the insecticide was removed from the foliage.

Figure 1 shows a comparison between the persistence of the diflubenzuron applied under similar circumstances, but in two forests with differing climates. A shorter period of persistence in the Atlantic forests can be clearly appreciated when contrasted

TABLE I Mean values ($n=6$) of the residual concentration of DFB (C_t) on foilage and standard deviations (SD) each sampling day of the conifer forest. Quality control of sampling and analysis: F , factor of the analysis of variance. Critical value of F to this distribution: $F(2,3,95\%)=9.552$. Limit of determination of DFB from pine needles: $DL=12 \text{ ng g}^{-1}$

t (days)	$C_t \pm SD$ (ng g ⁻¹)	F	t (days)	$C_t \pm SD$ (ng g ⁻¹)	F
1	1080 ± 18	3.095	86	182 ± 7	2.640
4	998 ± 19	1.404	93	155 ± 6	2.659
10	903 ± 16	1.063	100	138 ± 6	2.678
16	809 ± 15	2.595	107	118 ± 5	1.785
21	738 ± 13	1.419	116	101 ± 5	0.305
26	675 ± 13	2.412	125	87 ± 5	2.377
30	632 ± 10	0.771	135	74 ± 5	1.688
35	581 ± 15	2.748	144	62 ± 5	3.057
40	530 ± 15	2.485	153	52 ± 3	1.828
44	487 ± 11	0.586	163	44 ± 3	2.025
51	418 ± 10	0.510	174	30 ± 2	1.251
58	358 ± 11	3.014	185	22 ± 2	2.846
65	295 ± 12	2.177	196	13 ± 1	2.600
72	249 ± 7	2.257	203	(< DL)	
79	210 ± 7	2.841			

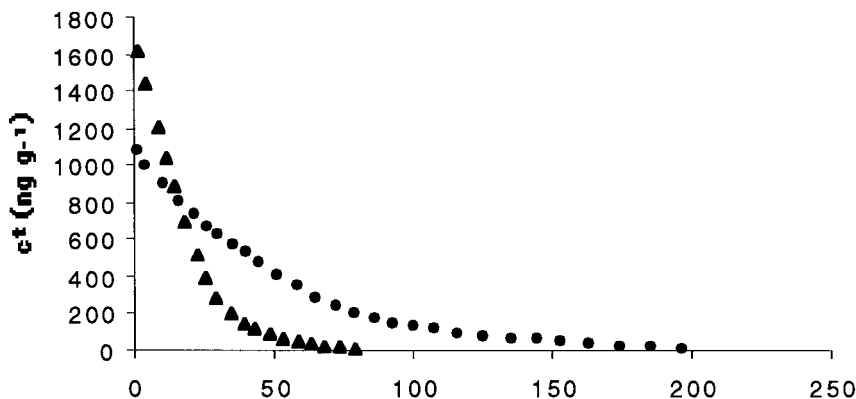


FIGURE 1 Persistence of diflubenzuron on foliage of the conifer forest. (●) Mediterranean-climate; (▲) Atlantic-climate [19].

to the Mediterranean forests (79 compared to 196 days), with a more rapid degradation in the first two months, expressed by the slope on the straight section of the persistence curve (-50.1 compared to $-10.9 \text{ ng g}^{-1} \text{ day}^{-1}$).

In order to validate the sampling protocol and the results of the persistence study, a statistical procedure based on analysis of variance [23] was applied. As shown in Table I, the F factor values were lower than the critical value of this parameter for this distribution each sampling day in the forest. The residual concentration of DBF can be represented by the mean value of the results obtained by analysing these samples. Furthermore, the reliability of the results was checked by calculating their standard deviations (Table I).

Influence of Weather and Environmental Conditions in the Persistence of Diflubenzuron on Foliage

The residual concentration of DFB can be presented as a first-order equation:

$$\log C_t = -0.0092t + 3.0689 \quad (3)$$

where C_t (ng g^{-1}) and t (days). The obtained values of $r^2 = 0.9946$ mean this first-order correlation is satisfactory to express the kinetic of DFB disappearance on foliage. This is according to the proposals of other authors [17,24].

Several parameters affect the persistence of diflubenzuron on pine leaves: photo-degradation (which can be estimated from the parameters of solar radiation, S , and temperature, T_m), hydrolysis and run-off from pine needle surfaces (related to rainfall, R , or relative humidity, H_R) in addition to other parameters such as the dosage and the conditions of its application [25,26]. The cumulative values of S , R and T_m were used to correlate the ecosystem meteorology to the residual concentration of the insecticide (C_t). Cumulative solar radiation (S_C) and rainfall (R_C) each sampling day (t) after application were calculated by adding up the daily values of (S) and (R) throughout this time interval (Δt). The cumulative temperature (Tm_C) in the same period (Δt) was calculated by averaging out the daily mean values of (T_m). Diverse multivariable linear regressions were applied to these data in order to obtain a mathematical correlation with the

experimental values of C_t . The best regression coefficient ($r^2=0.997$) was obtained when regressing $\log C_t$, R_C , S_C and Tm_C by the following expression:

$$\log C_t = 0.026Tm_C - 2.295 \times 10^{-5} S_C - 0.002R_C + 2.627 \quad (4)$$

where C_t (ng g^{-1}), R_C (mm), S_C (cal cm^{-2}) and Tm_C ($^{\circ}\text{C}$). No improvement in r^2 was obtained by including time and H_R in correlations. Figure 2 illustrates the correlation between DFB residual concentration and the meteorological variables described by Eq. (4). As can be observed, $r^2=0.997$ signifying that this empirical equation can be considered satisfactory to correlate the residual concentration of DFB (C_t) with meteorological variables R , S and T_m . According to these considerations, either the meteorological or forest characteristics have to be taken into account to determine the minimal dose of DFB needed to achieve the required phytosanitary effect.

The results obtained in the above mentioned study, which corresponded to a forest with an Atlantic climate [19] with similar arboreal density and height, gave the following correlation type, with $r^2=0.994$:

$$\log C_t = 0.065Tm_C - 5.410 \times 10^{-5} S_C - 2.970 R_C - 2.362. \quad (5)$$

This indicates a far greater influence of the rainfall parameter (R_C) in the degradation of diflubenzuron in the Atlantic forests when compared to the Mediterranean forests. The cumulative temperature (Tm_C) and solar radiation (S_C) parameters contribute to the phenomenon of degradation to the same extent in both climatic ecosystems.

As in the case of the Atlantic climate forests [19] 2,6-DFBA was the only metabolite detected on foliage. Table II shows the values of the concentrations of this compound each sampling day of the conifer forest together with the results of quality control of sampling and analysis. As can be observed in Fig. 3, 2,6-DFBA began to be detectable

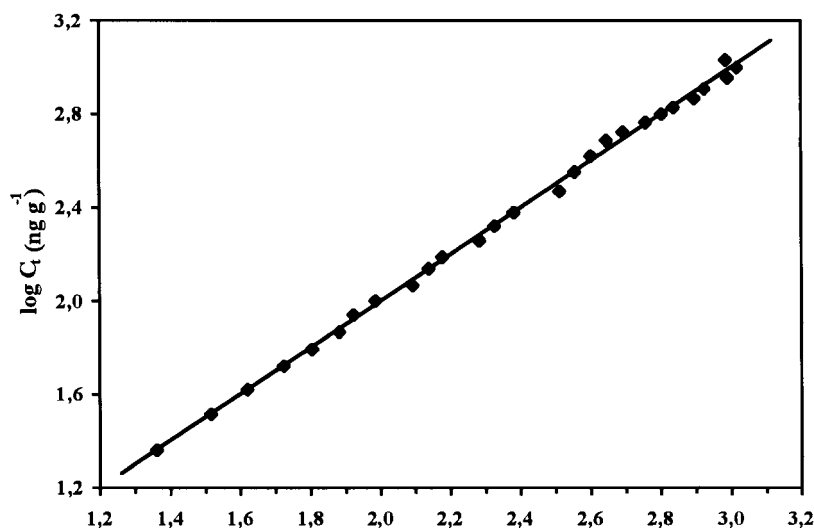


FIGURE 2 Empirical correlation between residual concentration of diflubenzuron (C_t) and meteorological variables: cumulative temperature (Tm_C), cumulative solar radiation (S_C) and cumulative rainfall (R_C).

TABLE II Mean values ($n=6$) of concentration of 2,6-DFBA (C_t) on foliage and standard deviations (SD) each sampling day of the conifer forest. Quality control of sampling and analysis: F , factor of the analysis of variance. Critical value of F to this distribution: $F(2,3,95\%)=9.552$. Limit of determination of 2,6-DFBA from pine-needles: DL = 35 ng g⁻¹

t (days)	$C_t \pm SD$ (ng g ⁻¹)	F
1	(<DL)	–
4	(<DL)	–
10	38.2 ± 6.7	2.844
16	71.5 ± 3.0	3.101
21	85.3 ± 3.6	2.947
26	(<DL)	–
30	(<DL)	–
35	45.1 ± 2.3	2.274
40	(<DL)	–
44	(<DL)	–

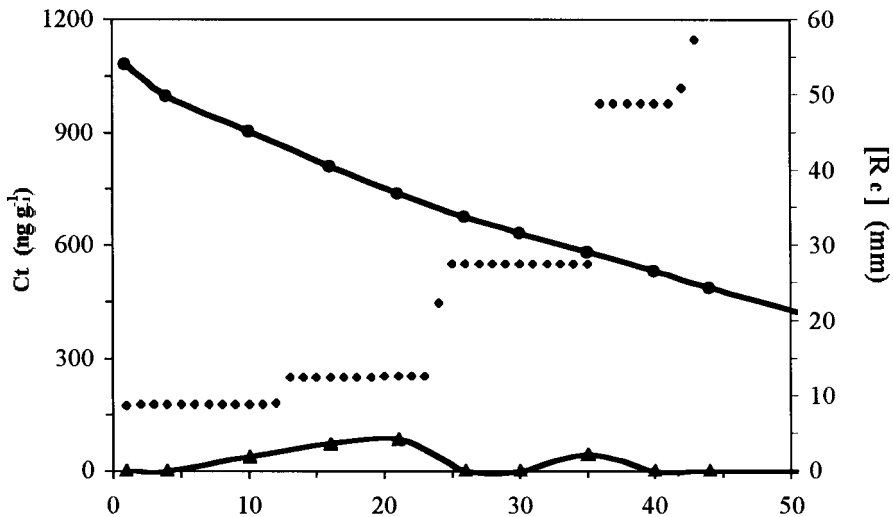


FIGURE 3 Variation of residual concentration of (●) DFB and (▲) 2,6-DFBA with time during the 50 early days. (◆) Values of cumulative rainfall (R_c) in this time period.

10 days after the aerial application of diflubenzuron and its concentration was increasing on pine-needle surface during the following week, as the concentration of DFB decreased. With the occurrence of the first rainfall upon the forest (24 and 25 days after application), the concentration of 2,6-DFBA on foliage was undetectable. Once again, 10 days later, coinciding with a dry period, this concentration on foliage was seen to be further increasing and 36 days after application, with the occurrence of the rainfall upon the forest, the concentration of 2,6-DFBA was undetectable (under 35 ng g⁻¹).

Previous studies carried out in greenhouses [19] have shown that hydrolysis was not the cause of degradation of diflubenzuron on foliage and thus, the possible influence of rainfall in the persistence of the insecticide could be due to the run-off of DFB from foliage. The other possible way of degradation of diflubenzuron [25] is

photodegradation. This occurs in two different ways: (i) producing 2,6-DFBA and 4-CA or (ii) forming 2,6-DFBA and 4-CPI. Following the same reasoning, the absence of 4-CA suggests that (ii) was the only possible way of degradation of diflubenzuron on foliage in this conifer forest. Thus, 2,6-DFBA appeared due to photodegradation of DFB and was later removed from pine needles by rainfall. Simultaneously formed 4-CPI is a very volatile compound and, therefore, does not persist on foliage [19,27]. This is apparently why 2,6-DFBA was the only metabolite detected in the persistence study. On the other hand, the absence of 2,6-DFBA throughout the first week after the aerial application of diflubenzuron was according with the previously reported predominance of other factors over the influence of the studies meteorological variables during the first 10–12 days after the application of the insecticide.

CONCLUSIONS

The proposed analytical methodology has enabled the measurement of the concentration of diflubenzuron and its main metabolites in conifer foliage after aerial application. The results obtained in the current study, in a Mediterranean climate forest, have been compared with other available studies of forests in an Atlantic climate. The present data indicate that diflubenzuron does not persist for prolonged periods (less than 196 days) in forestry substrates in a Mediterranean climate. However, this persistence is significantly greater than that found in Atlantic forests, in which persistence values of 72 days are obtained. Rainfall is the dominant influential factor in the persistence of the insecticide in both climates. The study suggests that photodegradation is the only possible way of degradation on conifer foliage and that this is made clear by the appearance of only one metabolite 2,6-DFBA.

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