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QuEChERS-Based Method for the Determination of Fipronil in Protein Baits and *Vespa velutina* Larvae by HPLC-DAD and GC-MS

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Abstract: Protein baits containing fipronil as a biocide have shown their effectivity as a control method for *Vespa velutina nigrithorax* (Lepeletier, 1836) in apiaries. This biocide is not selective for *Vespa velutina*, so it is important to use the minimum dose to inactivate a nest. Therefore, the aim of this work was the development of analytical methods for the determination of fipronil in protein baits for quality control purposes and in larvae of *Vespa velutina* to determine the biocide content after protein bait ingestion and to acquire knowledge on fipronil metabolism in larvae. For this purpose, a Quechers-based HPLC-PDA method was developed and validated for the determination of fipronil in both matrixes. Furthermore, a GC-MS method was developed for the analysis of fipronil and its metabolites in dead *Vespa velutina* larvae fed with a mash containing 0.01% fipronil. Quechers-based HPLC-DAD allowed for the determination of the fipronil content in baits. Fipronil and the metabolites fipronil sulfone and fipronil sulfide were identified by GC-MS in extracts of larvae fed with a protein mash containing 0.01% fipronil. The transformation of fipronil into fipronil sulfone inside the larvae and the high toxicity of this metabolite open the possibility to produce protein baits with lower biocide concentrations.

Keywords: QuEChERS; chromatography; fipronil; protein bait *Vespa velutina larva*; metabolism

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1. Introduction

Vespa velutina nigrithorax (Lepeletier, 1836), more commonly known as the Asian hornet, is a eusocial hymenopteran that was mistakenly introduced into France in 2004 [1–3]. Since its introduction, it has been spreading to different European countries such as Portugal, Italy, Belgium, and Spain, among others [4–9].

The Asian hornet has a life cycle similar to that of other social wasps/hornets [2]. This begins in spring, when the queens build the first nests (embryo or primary nests) in protected places, and with the hatching of the workers, the colony grows and can be composed of hundreds to thousands of individuals [10,11]. In most cases, with the arrival of summer, the colonies need to build a new nest in a larger space, which is called a secondary nest, reaching its maximum size in mid-to-late summer. This time is when massive attacks by hornets on apiaries and crops take place. This fact is due to the need for food with protein needed to feed the larvae, which is mainly provided by insects hunted by the workers, mostly honeybees (*Apis mellifera*, Linnaeus, 1758) [10], as well as sugars for the adults [12,13]. Finally, with the arrival of autumn, the mating period begins, followed by the death of the colony due to the lack of food and the decrease in temperature. In this period, the newly fertilised and overfed queens look for protected places where they can spend the winter in hibernation and start a new cycle the following spring [3,10,11,14].

The presence of this species outside its natural habitat and the lack of predators are causing damage in different sectors, such as the environmental sector, since it is a predator

of native insects, especially honeybees; the agricultural sector due to the destruction of fruit and vegetable crops; and public health [15,16].

The impact caused by this species has led to the development of different control methods to fight against it [17–20]. These methods can be divided into two main groups: those based on the destruction of the nests [21] and those that focus on trapping the hornets [22]. Within the second group are the attractant traps made from sugary ingredients and alcohol, which, although effective, are not selective for this species, and their uncontrolled use may cause biodiversity problems [23].

The high pressure and attacks on apiaries have led to the use of some additional control methods that help to minimise its impact, such as muzzles [24] and electrified traps [25]. Protein baits with biocide, which would be placed near the apiary, are included in this group of methods. The hornets would be attracted by baits instead of by honeybees and would bring them to the nest to feed the larvae, thus reducing the pressure on the apiary. Additionally, once introduced into the nest and fed to the larvae, these secrete a carbohydrate-rich fluid that they will provide to the adults by trophallaxis, inactivating the nests for several days as they cause the death of both larvae and adults. The efficacy of the protein baits with fipronil as a control method has been previously demonstrated in apiaries [26,27].

In the literature, the most commonly used biocidal baits are based on fish, meat, synthetic protein, or paraffin bases [28–30]. The different matrices can contain the biocide either free or encapsulated [31]. The majority of the protein baits commercially available for the control of the Vespidae family contain fipronil as a biocide in their composition [28,32–34].

Fipronil is a broad-spectrum biocide insecticide of the second-generation phenylpyrazole family. It is commonly used for pest control in both agriculture and animal products, such as flea control in dogs and cats [35]. The insecticide acts by blocking the chloride channels associated with gamma-aminobutyric acid (GABA) and glutamate (Glu) receptors in insects. It blocks neuronal inhibitory receptors, resulting in neuronal hyperexcitability caused by accumulation of the neurotransmitter GABA in the synaptic cleft, causing the death of the individual [36–38].

In the European Union, Commission Implementing Regulation N° 540/2011 of 25 May 2011 approved the use of active fipronil [39]. However, as it is not selective for *Vespa velutina*, it is important to use the minimum dose to inactivate a nest, killing larvae and adults, since eggs and operculate larvae would not be affected by the baits.

The metabolism of fipronil may vary depending on the species ingesting it. For example, in humans, fipronil is metabolised mainly in the liver by cytochrome P450 enzymes to fipronil sulfone, its main active metabolite, and to a lesser extent to fipronil sulfide [40–42], Figure 1. The toxicity of these metabolites can be prolonged as fipronil sulfone is more persistent than the biocide and can accumulate in the body. Elimination occurs via urine and faeces, although this may be slow, increasing the risk of toxicity if the person is repeatedly exposed to the biocide [35,37,43,44].

In insect larvae, as in humans, fipronil is mostly metabolised to fipronil sulfone and fipronil sulfide. These metabolites act in a similar way to fipronil by inhibiting GABA receptors in the nervous system [38], but due to their greater chemical stability and slower elimination, they remain in the body for longer, increasing their toxic action. They also have a high persistence in insect tissue, contributing to their insecticidal efficacy [43,45–48].

Due to the complex composition of protein bait matrixes and *Vespa velutina* larvae, the analysis of the biocide fipronil requires a previous treatment of the samples to extract the analyte of interest with the lowest number of interferences. QuEChER is one of the most widely used methodologies for the analysis of insecticides, pesticides, and other contaminants in complex matrices such as food or biological samples [49–53]. This procedure is based on a combination of reagents that allow for dispersive solid–liquid extraction and adsorption purification to obtain clean extracts and concentrated analytes from complex samples in an efficient way.

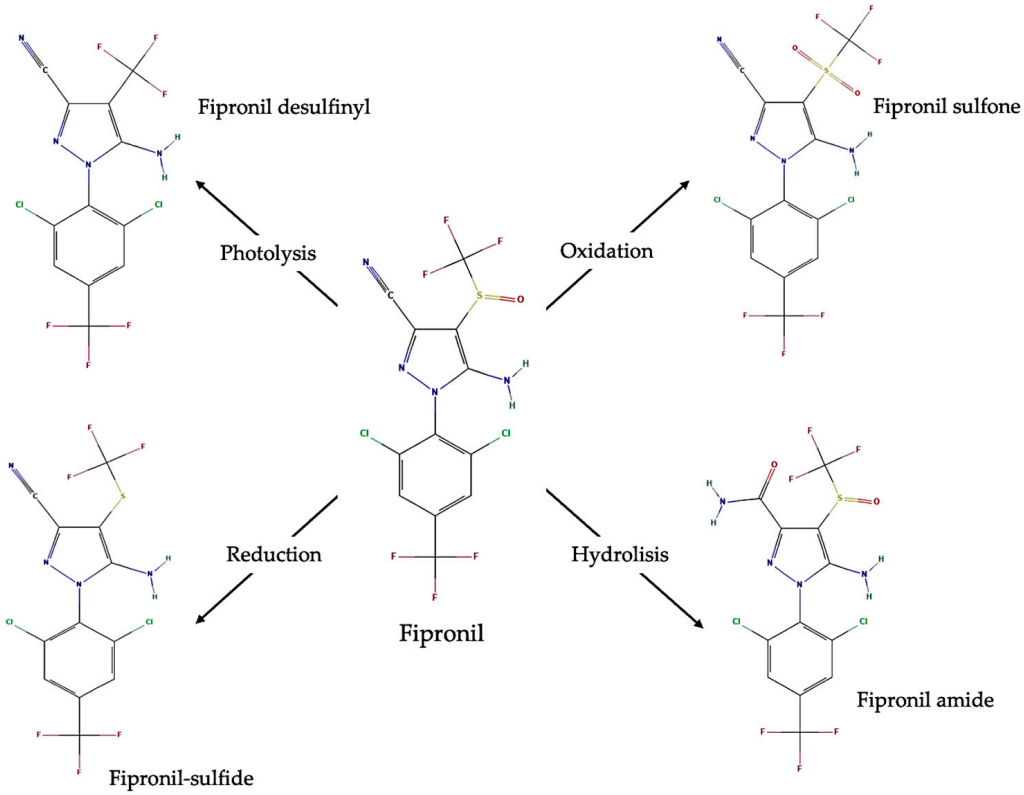

Figure 1. Fipronil and its metabolite (fipronil sulfone, fipronil sulfide, fipronil desulfinyl, and fipronil amide) structures. Image modified from pubchem.ncbi.nlm.nih.gov, accessed on 19 September 2024.

Extracts obtained from QuEChERS extraction are perfectly complemented by analytical methods such as chromatographic techniques.

High-performance liquid chromatography (HPLC) and gas chromatography (GC) coupled to mass spectrometry (MS) are the most commonly used techniques for the analysis of fipronil in food, textiles, water, soil, and sediments due to their high sensitivity and identification capacity [54–57]. However, liquid chromatography with ultraviolet detection (HPLC-UV) is also a widely used technique due to its rapidity and usefulness for the detection of compounds based on their UV-Vis spectra. This makes it a valid option for the analysis of fipronil and other biocides when the use of mass spectrometry is not necessary [26,58], since its sensitivity is enough to determine the expected concentrations.

The aim of this work was the development of analytical methods for the determination of the insecticide fipronil in protein baits for quality control purposes and in larvae of *Vespa velutina* to determine the biocide content after protein bait ingestion and to acquire knowledge on fipronil metabolism in the larvae of this invasive species. For this purpose, an analytical method was developed and validated by HPLC-PDA, a previous extraction procedure with QuEChERS, for the determination of fipronil in protein baits and larvae of *Vespa velutina*. Furthermore, a GC-MS method was developed for the analysis of fipronil and its metabolites in dead *Vespa velutina* larvae fed with a mash containing 0.01% fipronil.

2. Materials and Methods

2.1. Protein Baits and Vespa velutina LARVAE Samples

Vespa velutina larvae were obtained from 2 secondary nests collected from Bizkaia (Spain) and supplied by Basalan (Provincial Council of Bizkaia, Lezama, Spain). The nests were transported in transparent plastic boxes with small holes to allow the hornets and larvae to breathe. After arrival at the laboratory, the hornets were anaesthetised by adding diethyl ether (99.7%) (Panreac Applichem, Barcelona, Spain) in order to obtain the combs that contained the larvae.

Once asleep, the hornets and the external cover of the nest were removed, and the three combs obtained with the larvae were separated and placed individually in transparent boxes.

Three protein baits containing 0.01% (*w*/*w*) fipronil as biocide and another protein bait without biocide, which was used as a blank, were supplied by the company DTS-OABE, S.L. (Orozko, Bizkaia, Spain). The validation method and preparation of the protein mashes were performed with blank protein baits. The baits were stored at −80 °C until use.

2.2. Quality Control (QC) Samples Preparation

The quality control (QC) solutions for fipronil analysis and method validation in protein baits were prepared by dilution of stock solution of fipronil in mobile phase at 3 concentration levels: LLOQ (low concentration, 0.5 mg/L), MLOQ (medium concentration, 8 mg/L), and ULOQ (upper concentration, 16 mg/L). For the analysis of fipronil in *Vespa velutina* larvae, the QC samples were fipronil solutions diluted with mobile phase at the concentration levels of LLOQ (low concentration, 0.5 mg/L), MLOQ (medium concentration, 2 mg/L), and ULOQ (upper concentration, 4 mg/L).

2.3. In Vivo Assays with Larvae

Mashes for feeding the larvae were prepared from the blank protein bait. For the preparation, 6 g of protein bait without biocide was weighed in a CP224S analytical balance (±0.0001 g) from Sartorius (Madrid, Spain) and homogenised manually with 15 mL of Milli-Q water (18.2 MΩcm) (Milli-Q Advantage A10 System, Merck Millipore, Darmstadt, Germany) until a mixture with the consistency of mash was obtained. This was divided in two, and a 0.01% (*w*/*w*) concentration of technical-grade fipronil supplied by D+S-OABE, S.L. was added to one of them and homogenised. The other mash without biocide was considered as a blank. Both were stored in the freezer until use.

Two of the three combs with larvae were fed with a protein mash containing 0.01% (*w*/*w*) of the biocide fipronil using a Pasteur pipette. Each larva of one of the groups received a drop of mash (0.0116 ± 0.0008 g), a group named L1D (Larvae 1 drop). The larvae of the other group were placed in contact with the mash for 20 s, named L20S (Larvae 20 s), in order to test the ingestion method's repeatability. The remaining comb received a protein mash without biocide and was used as a control (LC) for normal larval activity (Figure 2).

Figure 2. Control samples of *Vespa velutina* larvae.

After feeding, the activity of the larvae was checked periodically. For this purpose, a Pasteur pipette with food was placed near the mouth of the larvae. If the larvae responded by taking the food and their movements were similar to those in the control group, these larvae were considered to have normal activity. However, those that did not respond to food and were not dead were considered inactivated larvae. Once all larvae were dead, the spent time was counted and the larvae were removed from the cells, placed in a storage container, and frozen at −80 until their analysis by HPLC-PDA and GC-MS. The total numbers of larvae stored were 50 and 62 for L20S and L1D, respectively. Additionally, 32 control larvae were also frozen for later use as control larvae and for validation of the method.

2.4. Sample Treatment

To carry out the extraction procedure, 1 g of protein bait and each larva (mean weight: 0.34 g) were weighed with an analytical balance. In order to compare the behaviour of the two larva groups fed with the different procedures described above, 50 larvae of each group were treated and analysed.

The protein bait samples were placed in a 50 mL Falcon tube, and 9 mL of HPLC grade acetonitrile (ACN) from Scharlab (Barcelona, Spain) was added. They were then homogenised by dispersion using an Ultraturrax T25 from IKA (Staufen, Germany) at 25,000 rpm for 2 min.

The larvae were placed in 2 mL vials containing seven zirconium balls 3 mm in diameter, and 1 mL of ACN was added. The mixtures were homogenised using the Tisssuelyser Precellys 24 homogeniser from Bertin Instruments (Montigny-l-Bretonneus, France) at 4000 rpm, and 3 cycles of 20 s with 30 s of rest were performed between cycles. The mixture obtained was made up to a final volume of 9 mL of ACN.

To the homogenates of both matrices, 650 mg of the components of the QuEChERS first extraction step from Agilent Technologies (Santa Clara, CA, USA) was added. The mixtures were shaken for 1 min on a rotary shaker at maximum speed and centrifuged (15 min; 5000 rpm; 15 °C) in an Eppendorf 5804 R centrifuge (Hamburg, Germany). Next, reagents from the QuEChERS clean-up step were added to the supernatants to remove any remaining organic acids, fatty acids, sugar, and water. The mixture was shaken and centrifuged. To precipitate the possible remaining lipids, the supernatants were frozen at −20 overnight. The supernatants were filtered (0.45 µm) and evaporated to dryness with a nitrogen flow at 50 °C using a Zymark TurvoVap® evaporator (Hopkinton, MA, USA).

The protein bait extracts were reconstituted with 250 µL of ACN, filtered (0.45 µm), transferred to 300 µL inserts for 2 mL vials, and injected into the HPLC-PDA.

Larval extracts were reconstituted in 250 µL Scharlab ethyl acetate (AcOEt), placed in 300 µL inserts for 2 mL vials, and injected into the GC-MS.

2.5. Chromatographic System and Conditions

2.5.1. HPLC-PDA

A 2695 HPLC system coupled to a 996 photodiode array detector from Waters (Milford, MA, USA) was used for the biocide detection. The chromatographic separation was carried out using an HPLC C18 Supelco ® ABZ+Plus (25 cm × 4.6 mm, 5 µm) column and a C18 pre-column (4 × 3.0 mm) from the manufacturer Sigma Aldrich (St. Louis, MI, USA). Ultrapure Milli-Q water with 0.1% of trifluoroacetic acid (TFA) 99% supplied by Scharlab (A), and 100% HPLC grade ACN (B) were used as mobile phases. The chromatographic peaks were acquired and integrated by Empower 3 software from Waters. The conditions of HPLC-PDA analysis for fipronil are shown in Table 1.

Table 1. HPLC-PDA conditions for the analysis of fipronil in protein baits and *Vespa velutina* larvae.

Parameter	Conditions
Column	HPLC C18 ABZ+Plus (25 cm × 4.6 mm, 5 µm)
Column temperature (°C)	40
Elution mode	Isocratic
Flow rate (mL/min)	1
Injection volume (µL)	10
Mobile phase	35% aqueous phase (A) (H_2O, 0,01% TFA) 65% organic phase (B) (100% ACN)
Autosampler temperature (°C)	4
Analysis time (min)	12
Wavelength (nm)	277

2.5.2. GC-MS

Gas chromatographic analysis was performed with an Agilent 6890 N Network gas chromatography system coupled to a CTC-PAL 120 autosampler (Zwingen, Switzerland). A HP-5MS UI column (30 m × 0.25 mm ID × 0.25 µm) from Agilent Technologies was used for chromatographic separation. A mass spectrometric detector, Agilent 5973-N, coupled to the chromatographic system was used. Table 2 shows the conditions of GC-MS analysis.

Table 2. GC-MS conditions for the analysis of fipronil and metabolites in *Vespa velutina* larvae.

	Parameter	Conditions
GC	Carrier gas	Helium 1 mL/min (constant flow)
	Column	HP-5MS UI (30 m × 0.25 mm ID × 0.25 µm)
	Injection temperature (°C)	280
	Temperature gradient	Initial temp.: 100 °C for 1 min Ramp: 10 °C/min to 150 °C; 25 °C /min to 175 °C; 10 °C /min to 250 °C and hold 4 min
	Scan time (min)	17.5
MS	Transfer line temperature (°C)	300
	Solvent delay (min)	3
	Mode	SCAN (range: 40 to 400 *m*/*z*) SIM *m*/*z* ions: - Fipronil: **367**; 370;371 - Fipronil sulfone: **383**; 385; 384 - Fipronil sulfide: **351**; 353; 355 - Fipronil desulfinyl: **388**; 335; 334
	Dwell time (ms)	50
	Detector temperature (°C)	300

m/*z* quantifier ions are written in bold.

2.6. Recovery of the Sample Treatment

The recovery study of the treatment procedure was performed by analysing blank samples spiked with fipronil before and after sample treatment.

For this purpose, 3 replicates of blank protein bait were spiked with 10 mg/L of the biocide before the complete QuEChERS procedure. Another blank sample was spiked with 10 mg/L of the biocide after finishing the sample treatment. The extracts obtained were analysed by HPLC-PDA following the chromatographic conditions shown above. The obtained chromatographic peak areas were interpolated on the calibration curve, and the percentage recovery was calculated as the ratio between the two concentrations obtained.

2.7. HPLC-PDA Analytical Method Validation

The developed methods for fipronil analysis in protein baits and *Vespa velutina* larvae were validated following the criteria established by the SANTE guide of the European

Union for the analysis of pesticide residues in food [59]. The validated parameters were matrix effect, linear range, limits of quantification and limits of detection, and precision. Additionally, the accuracy parameter was validated according to the Food and Drug Administration (FDA) guide [60].

2.7.1. Selectivity

Selectivity was tested to check possible interfering compounds at the retention time of fipronil in the samples. This parameter was evaluated by comparing the chromatograms of the extracts obtained from the extraction of blank protein bait and control larvae samples with that corresponding to a 0.5 mg/L fipronil standard solution (LLOQ).

2.7.2. Matrix Effect and Linear Concentration Range

The matrix effect was determined by comparing the slopes of two calibration methods: external prepared in mobile phase and matrix-matched calibration for protein baits and larvae. A slope ratio around 1 was considered to be no matrix effect. In this case, validation of the analytical method would be carried out using external calibration.

To study the linear concentration range, calibration curves were constructed, taking into account the expected fipronil concentration in protein baits. Fipronil concentrations range between 0.5 to 16 mg/L for protein baits. The concentration range tested for fipronil larvae analysis was lower than that of protein baits, considering that the larvae were fed with a very small amount of mash containing fipronil. A calibration curve in a concentration range of 0.5–4 mg/L of fipronil for larvae analysis was built, and the data chromatographic peak area (mAU)-concentration of the biocide were treated by the linear regression method. Correlation coefficients (R^2) and residuals were used as criteria for linearity.

2.7.3. Limits of Detection and Quantification

The concentrations corresponding to the lower limits of detection (LLOD) and lower limits of quantification (LLOQ) were calculated by injecting 10 blank sample extracts into the chromatographic system. The mean chromatographic peak area plus 3 and 10 times the standard deviation for LLOD and LLOQ (Equations (1) and (2)), respectively, was interpolated in the calibration curves to obtain the concentrations corresponding to these limits. The reproducibility and accuracy of the LLOQ were also calculated in order to meet the criteria of validation guides.

$$\text{LLOD} = \overline{y} \pm 3 \cdot s_y \quad (1)$$

$$\text{LLOQ} = \overline{y} \pm 10 \cdot s_y \quad (2)$$

2.7.4. Repeatability and Accuracy

The intra- and inter-day repeatability and accuracy of the method of fipronil were determined using five replicates of the three QCs: LLOQ, MLOQ, and ULOQ.

The results were expressed in percentages of relative standard deviation (%RSD) and relative error (%ER) for repeatability and accuracy, respectively. The inter-day analyses were carried out on 3 different days: day 0, 5 days, and 12 days.

2.8. Fipronil Content in Protein Baits and Vespa velutina Larvae by HPLC-PDA

The obtained extracts of three protein baits containing fipronil as biocide and the larvae of *Vespa velutina* were analysed in triplicate by HPLC-PDA. A blank extract was injected between the sample groups to check the absence of contamination in the chromatographic system due to sample analysis.

2.9. Metabolism of Fipronil in Vespa velutina Larvae

GC-MS was applied to study the metabolism of the biocide fipronil in *Vespa velutina* larvae. The extracts obtained from the extraction of each larva with the QuEChERS method were used for this study.

The NIST14 spectra library from Agilent Technologies was used for the identification of fipronil and its metabolites, fipronil sulfone, fipronil sulfide, fipronil desulfinyl, and fipronil amide. MassHunter (version 6.0) software from Agilent Technologies was used for the data acquisition and integration of chromatographic peak areas.

3. Results and Discussion

3.1. In Vivo Assasys with Larvae

After 24 h of the ingestion of mash containing fipronil, larvae from both groups, L1D and L20S, showed no response to feeding stimulation and thus were considered inactivated, since they were not dead and abdominal movements were observed. The total mortality was observed after 48 h from the mash administration, and the larvae turned a blackish colour. Furthermore, some larvae fed with 0.01% of biocide showed a swollen aspect. Control larvae did not present changes in appearance, and their activity and response to food were normal.

The results of this study regarding the activity and changes in appearance of the larvae corroborate those obtained in the previous in vivo assays with larvae carried out by this research group [26].

3.2. Recovery of the Sample Treatment

The recovery percentage of fipronil for both matrixes' protein baits and larvae extracted using QuEChERS methodology was 75 ± 5%., which meets the established validation criteria for this parameter (range of mean recoveries: 60–120% for concentrations lower than 0.01 mg/kg).

3.3. HPLC-PDA Analytical Method Validation

Under the chromatographic conditions listed in Table 1, a chromatographic peak was obtained for fipronil at a retention time of 5.8 ± 0.1 min, as shown in Figure 3.

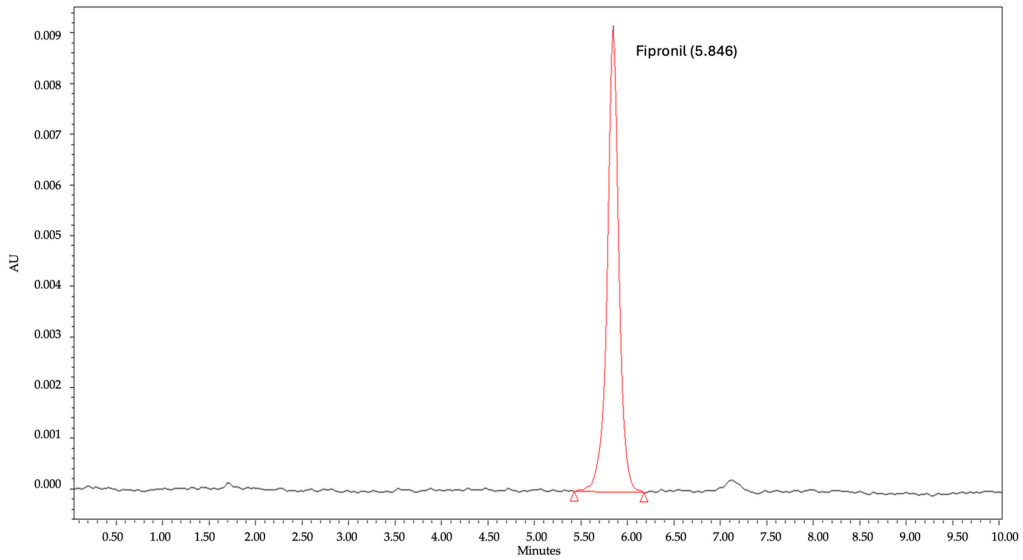

Figure 3. Chromatogram of a 10 mg/L fipronil solution in mobile phase under the chromatographic conditions given in Table 1.

3.3.1. Selectivity

Extracts of blank protein baits and control samples of *Vespa velutina* larvae showed no chromatographic peaks at the retention time of the biocide. Thus, the selectivity of the method for both matrices was satisfactory. Figure 4 shows the absence of interferences from the extracts of both matrixes at the retention time of fipronil.

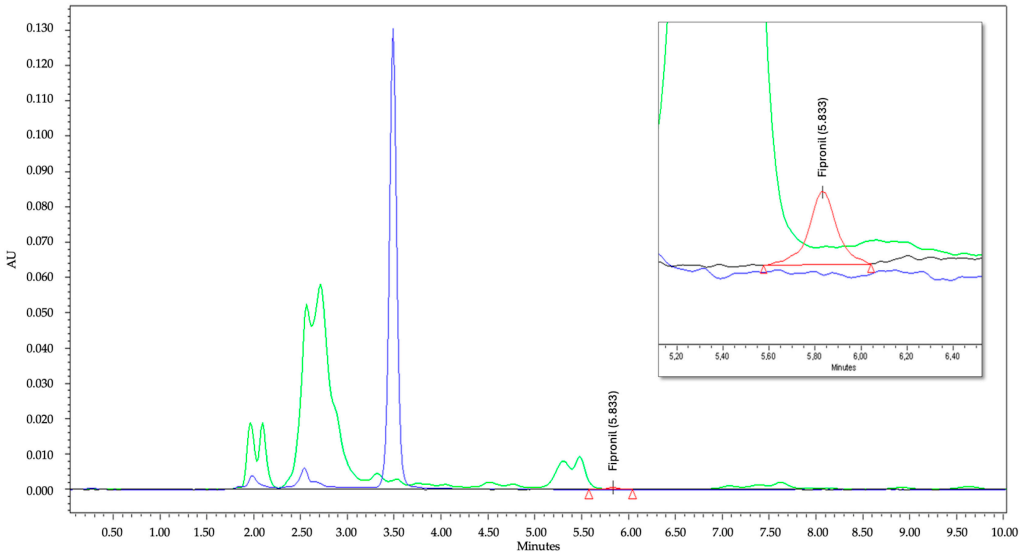

Figure 4. Chromatograms of a blank protein bait extract (blue line), a control larva extract (green line), and a standard fipronil solution at a concentration of 0.5 mg/L (black line). The integration of fipronil chromatographic peak is marked with a red line.

3.3.2. Matrix Effect and Linear Concentration Range

No differences were obtained in the slopes of both calibration methods for protein bait and larvae of *Vespa velutina*; the ratio was close to unity at 0.94 and 0.95, respectively. Therefore, the external calibration was used for the rest of the validation and quantitative analysis. Correlation coefficients greater than 0.99 and the obtained homoscedastic residuals confirmed the linearity of chromatographic area-fipronil concentration for both matrixes in the concentration ranges measured. The linear concentration ranges, together with the regression equations and ratio slopes calculated for fipronil in the two matrixes, are collected in Table 3.

Table 3. Linear concentration range, regression equations, and slope ratios for external calibration and matrix-matched calibration obtained for protein baits and *Vespa velutina* larvae for the analysis of fipronil.

Calibration Method	Linear Concentration Range (mg/L)	Regression Equations	Slope Ratio
External Protein-matched	0.5–16	$6851x - 1812$; $R^2 = 0.999$ $6458x + 86$; $R^2 = 0.999$	0.94
External Larvae-matched	0.5–4	$7745x + 747$; $R^2 = 0.999$ $7352x - 1586$; $R^2 = 0.998$	0.95

3.3.3. Limits of Detection and Limits of Quantification

The LLOD and LLOQ calculated using blank samples for both matrices were 0.25 and 0.5 mg/L, respectively. The collected values met the criteria established by the validation guidelines.

3.3.4. Repeatability and Accuracy

The intra-day and inter-day repeatability and accuracy values calculated for the 3 QC concentration levels (LLOQ, MLOQ, and ULOQ), expressed as relative standard deviation percentage (RSD%) and relative error percentage (ER%), are shown in Table 4.

Table 4. Intra-day and inter-day repeatability and accuracy, expressed in terms of RSD% and ER% obtained for fipronil analysis in both matrixes.

		Protein Baits			***Vespa velutina* Larvae**		
		LLOQ	**MLOQ**	**ULOQ**	**LLOQ**	**MLOQ**	**ULOQ**
Fipronil concentration (mg/L)		0.5	8	16	0.5	2	4
RSD%	**Intra-day**	5.0	1.0	0.8	4.7	1.6	1.2
	Inter-day	5.2	1.6	1.3	7.6	2.9	2.7
ER%	**Intra-day**	18.9	0.7	0.9	16.3	4.7	4.3
	Inter-day	19.0	0.7	0.2	16.7	4.8	4.4
RSD % SANTE criteria ER % FDA criteria		≤20	≤15		≤20	≤15	

The results indicated that the analytical method was repeatable and accurate both intra- and inter-days, considering the acceptability criteria of the European Union SANTE and the Food and Drugs (FDA) validation guidelines.

3.4. Fipronil Content in Protein Baits and Vespa velutina Larvae by HPLC-PDA

Figure 5 shows the chromatograms obtained for protein bait samples. The concentrations obtained for the protein baits supplied by the chemical company are shown in Table 5, expressed as the mean value of the percentage of fipronil per gram with a confidence level of 95%. The fipronil content obtained by the HPLC-DAD method is in total agreement with that reported by the company. This confirms the reliability of the analytical method developed for quality control assays.

Table 5. Fipronil concentration expressed in percentage per gram at 95% confidence level.

Bait Samples	**% of Fipronil/g Bait**
1	0.0104 ± 0.0004
2	0.0102 ± 0.0004
3	0.0102 ± 0.0001

However, the analysis of fipronil content in each larva fed with the mash containing 0.01% (*w*/*w*) of fipronil could not be determined, since the sample concentrations were below the LLOQ.

In order to assess the capacity of the HPLC-DAD method for the determination of fipronil in larvae, a pool of 7 larval extracts was prepared and injected into the chromatographic system. The analysis allowed for the quantification of fipronil in the larval pool (Figure 5). A concentration range of 1.49×10^{-4} mg to 3.47×10^{-4} mg of fipronil per larva was found. Nevertheless, a new chromatographic peak was observed in the analysis of the larvae pool, which eluted at longer retention times than fipronil and had not been obtained in the control larva samples. The UV-Vis spectrum of this chromatographic peak could have belonged to some fipronil metabolites [41,61–63].

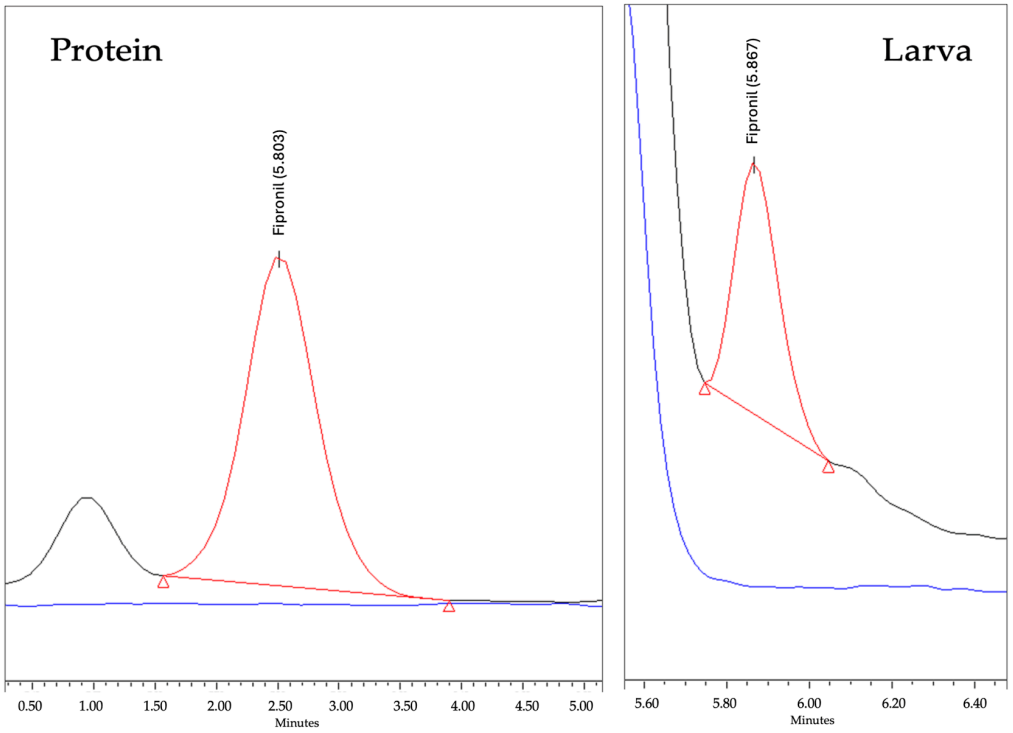

Figure 5. Chromatograms of a protein bait sample compared with a blank protein sample (**left**) and a group of 7 larvae sample and a blank sample of larvae (**right**).

Figure 6 shows the characteristic UV-Vis absorption spectrum of fipronil with its absorption bands around 200 and 280 nm, as well as the spectrum belonging to the new chromatographic peak observed with absorption bands at 195 and 270 nm. A shift of fipronil absorption bands towards lower wavelengths can be observed, as well as a significant increase in absorbance in the absorption band around 200 nm. This could be the result of changes in the fipronil structure giving rise to transformation compounds.

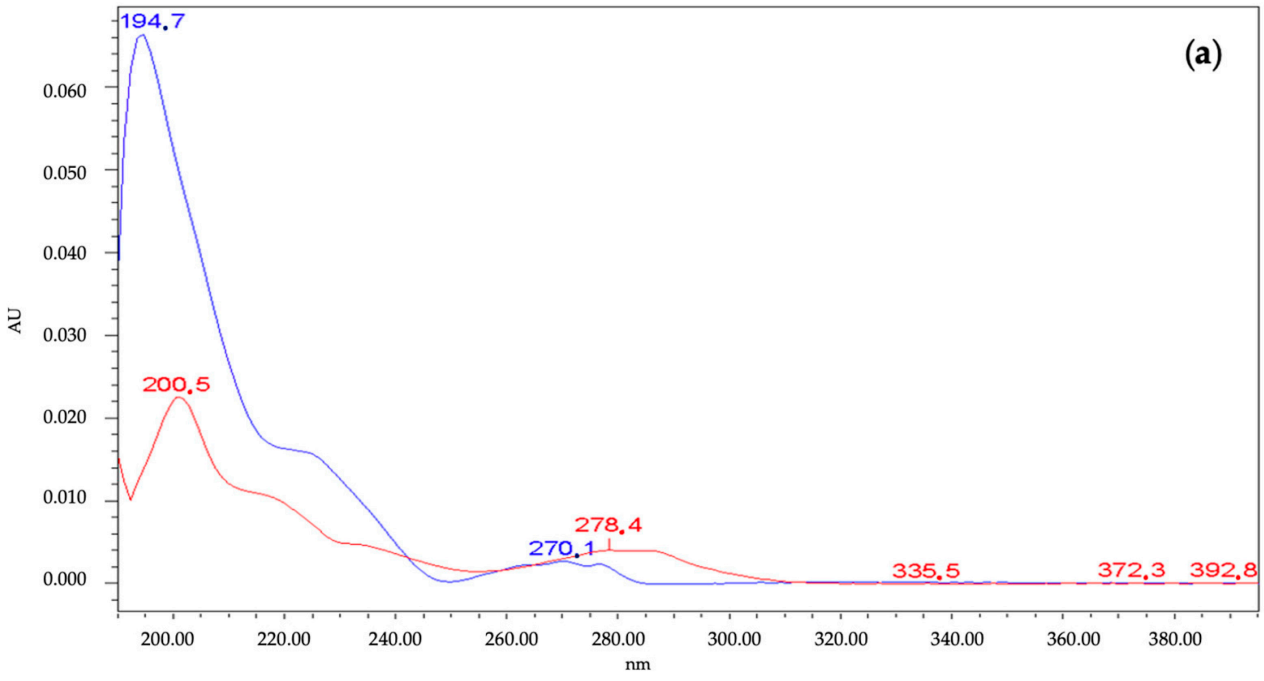

Figure 6. *Cont.*

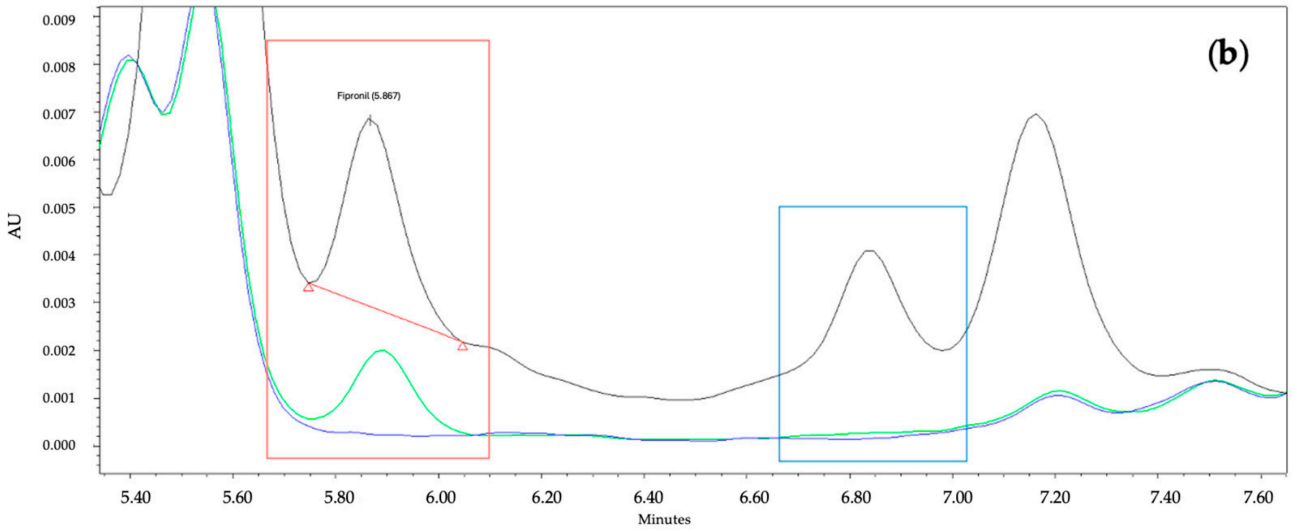

Figure 6. UV-Vis absorption spectra for the chromatographic peaks corresponding to fipronil (red square) and to the unknown compound (possible degradation/metabolite of fipronil (blue square) (**a**). Chromatogram of a pool of 7 *Vespa velutina* larvae (black), blank larva spiked with 2 mg/L of fipronil (green), and blank larva (blue) (**b**).

3.5. Metabolism of Fipronil in Vespa velutina Larvae

The low fipronil concentration found in larva extract could be attributed to both excretion processes and metabolism of the biocide. Therefore, we decided to analyse the larva extracts using an analytical technique with a higher level of sensitivity. GC-MS was applied to see if this analytical technique was able to analyse fipronil in each larva and give information on fipronil metabolism in *Vespa velutina* larvae.

Once the optimal temperature gradient for fipronil determination by GC-MS was chosen, a standard fipronil solution (2 mg/L) and a larva extract were injected into the system. Chromatograms were acquired in SCAN and SIM mode using the 367, 383, 351, and 388 m/z ions as quantifiers for fipronil and its metabolites, fipronil sulfone, fipronil sulfide (F. sulfide), and fipronil desulfinyl (F. desulfinyl), respectively [56,57,64]. The most characteristic m/z qualifiers were also acquired for each analyte.

As can be seen in Figure 7, three chromatographic peaks were obtained for the larva extract. Two chromatographic peaks at 10.34 (1) and 11.6 (3) minutes appeared together with fipronil (2), which corresponded to the metabolites fipronil sulfide and fipronil sulphone, respectively. It is important to note that the metabolites fipronil desulfinyl and fipronil amide were not identified, probably because they were not detectable, since they are minor metabolites formed by photodegradation and hydrolysis, respectively [37,57].

Once fipronil and its metabolites were identified, 50 extracts of each sample group, L1D and L20S, were injected into the GC-MS system, and chromatograms were acquired in SIM mode.

For individual larva samples, the percentages of the chromatographic peak areas of each analyte with respect to the total area obtained for the three chromatograms were calculated to assess the transformation procedure, which suffered fipronil in the larva. Prior to the calculation of the percentages, areas were normalised with the weight of each larva to avoid possible biases and to ensure an accurate representation of the relative amount of each analyte.

As can be seen in Figures 8 and 9, for the majority of the samples, fipronil sulphone had the highest percentage, showing percentages higher than 80%. In addition, the results indicated that the amounts of fipronil and fipronil sulfide were inversely related to fipronil sulfone. However, fipronil sulfide percentages remained practically stable in all samples, except in those where the fipronil percentage was higher. Regarding the two methods of

feeding the larvae, that in which the larvae were fed with the protein mash for 20 s showed more stable values.

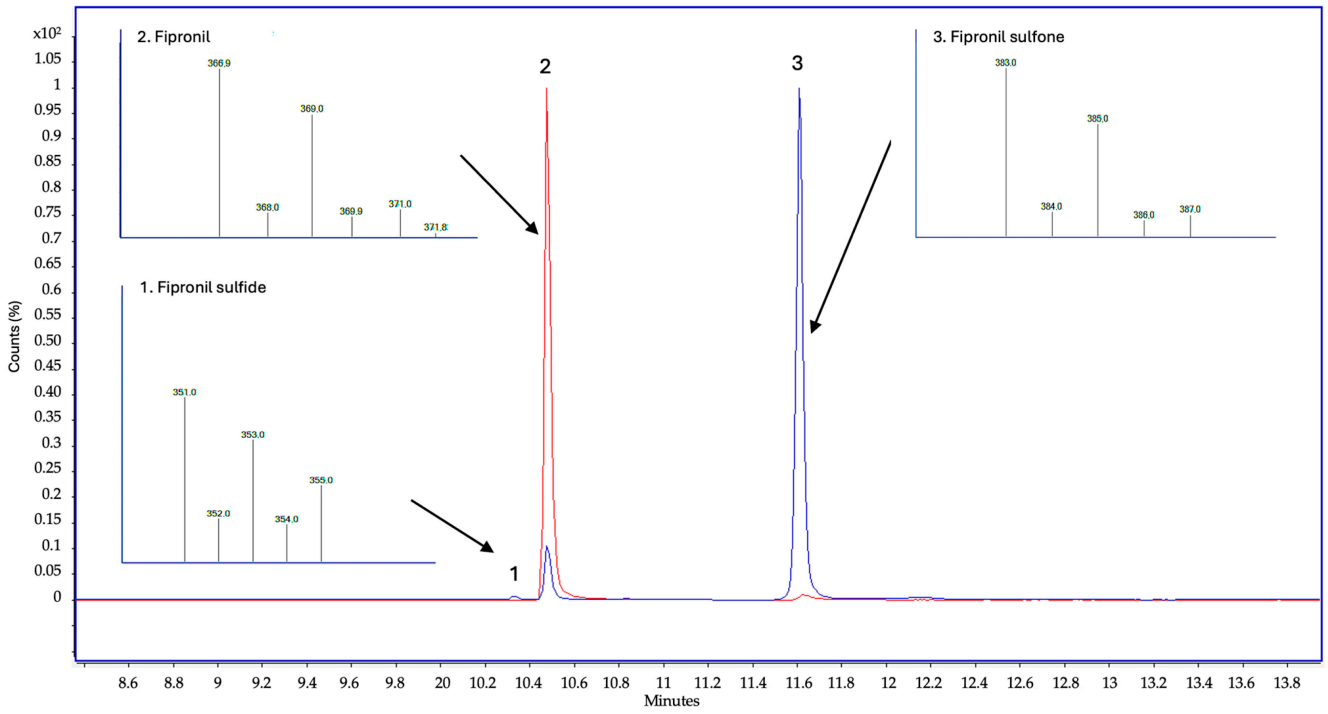

Figure 7. SIM mode GC-MS chromatograms of a standard solution of fipronil at a concentration of 2 mg/L (red line) and an extract of larva feed with a mash containing fipronil 0.01% *w*/*w* (blue line).

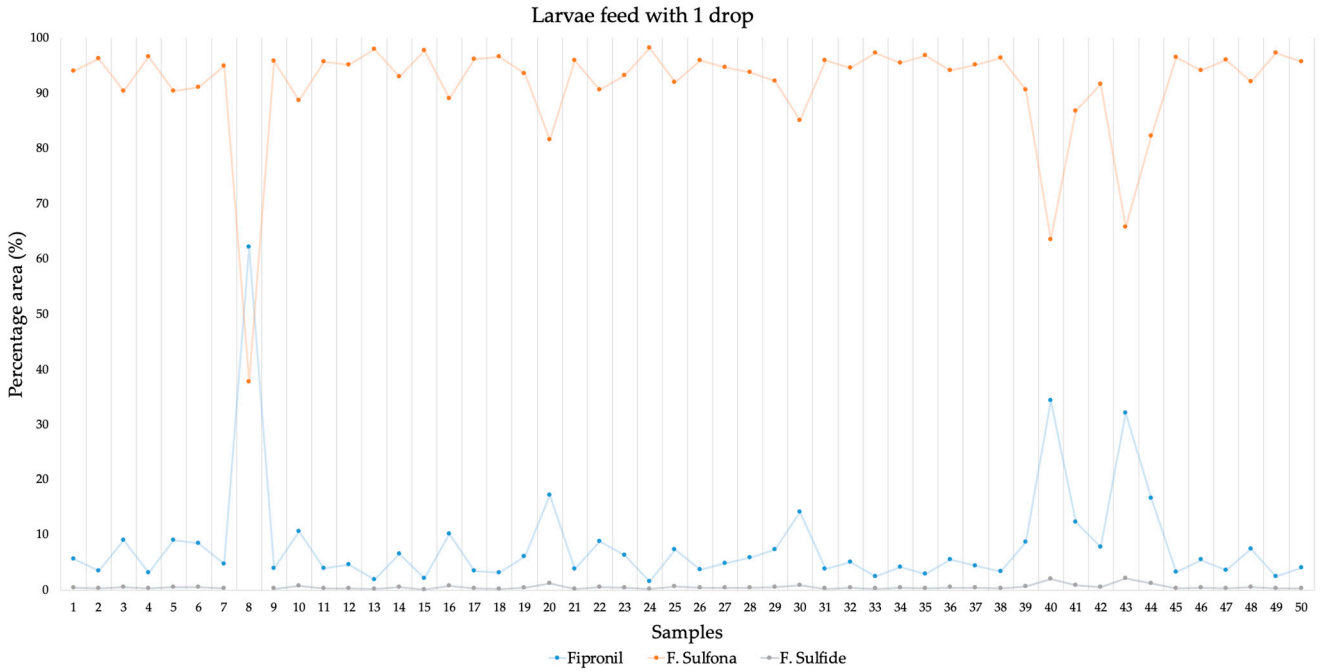

Figure 8. Variation in percentages of the chromatographic peak areas of the analytes fipronil (blue), F. sulfone (orange), and F. sulfide (grey) for the extracts of samples of larvae fed with a drop of protein mash containing 0.01% fipronil.

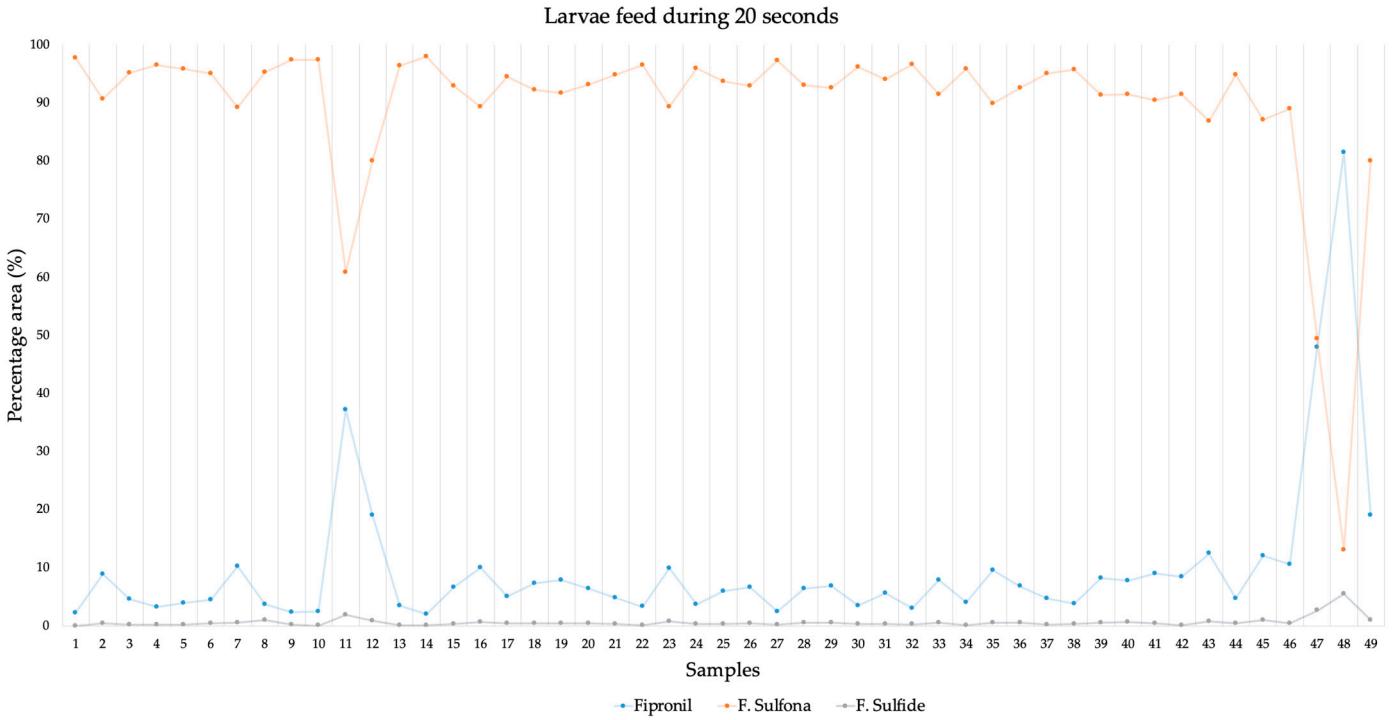

Figure 9. Variation in percentage of the chromatographic peak areas of the analytes fipronil (blue), F. sulfone (orange), and F. sulfide (grey) for the extracts of the samples of larvae fed with the protein mash containing 0.01% fipronil for 20 s.

These results are of particular interest for establishing the adequate doses of the protein baits as a control method for the invasive species *Vespa velutina*. The transformation of fipronil into fipronil sulphone could lead to a breakthrough in reducing the amount of biocide used in baits. This can be justified due to the higher toxicity of the two metabolites fipronil sulfone and fipronil sulfide than fipronil itself [26,48,65,66].

4. Conclusions

The analytical method developed using QuEChERS-based HPLC-PDA has proven to be selective, repeatable, and accurate according to SANTE and FDA guidelines for the determination of the biocide fipronil in protein baits and *Vespa velutina* larvae matrixes.

The fipronil concentration obtained in protein baits was in accordance with that certified by the chemical company. Therefore, it demonstrated the capacity of the analytical method for quality control purposes.

The inactivation of *Vespa velutina* larvae was effective after 24 h of feeding with protein mash containing 0.01% (*w*/*w*) of fipronil. The total mortality of the larvae was obtained after 48 h.

The sensitivity of the HPLC-PDA did not allow us to determine the fipronil content in an individual larva, but the concentration of fipronil in a pool of larvae was calculated to be in the range of 1.49×10^{-4} mg to 3.47×10^{-4} mg/larva.

Fipronil and two of its metabolites, fipronil sulfone and fipronil sulfide, have been identified in extracts of *Vespa velutina* larvae fed with a protein mash containing 0.01% fipronil by GC-MS.

The high transformation of fipronil into fipronil sulfone inside the larvae opens up an avenue for producing protein baits with lower concentrations of the biocide, knowing the highest toxicity of fipronil sulfone. This decrease obeys the demand of reducing the fipronil dose in baits because this biocide is non-selective for this invasive species.

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Data Availability Statement: The data presented in this study are available upon request from the corresponding author due to privacy reasons.

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