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ARTICLE

Development and Validation of an Analytical Method for the Determination of Fipronil and its Degradation Products in 28 Organic and Regular Honey Samples by GC-ECD

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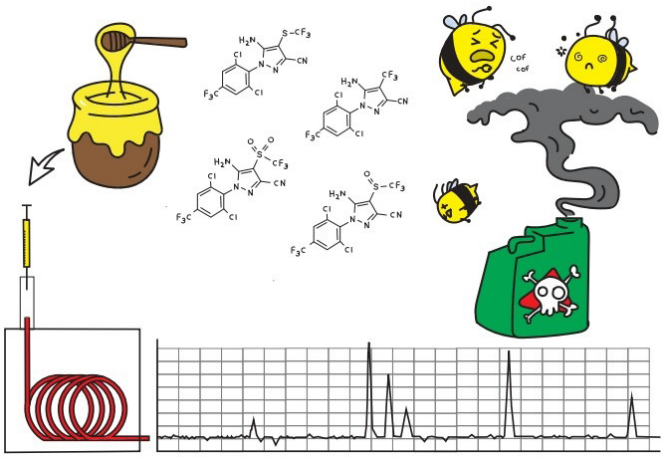

Recently, there has been a worldwide problem of increased bee mortality (Colony Collapse Disorder) and the intensive use of pesticides is suspected as one of the causes. Honey samples are one of many indicators to assess bee exposure to pesticides. In this work, a method for the simultaneous analysis of the pesticide Fipronil and its degradation products in honey samples by gas chromatography with electron capture detector (GC-ECD) is presented and validated. The extraction procedure was investigated using C18-SPE with different solvents and methanol showed the best performance. The analytical quantification was performed by internal standard matrix-matched calibration, which resulted in analytical curves presenting correlation coefficients higher than 0.99. The proposed method was validated with good results, such as recoveries around 70 – 99%, limits of detection and quantification bellow 0.014 and 0.072 μg mL^{-1}, respectively, and relative standard deviations below 7%. The method is simple, effective and was successfully applied to 28 commercial honey samples, regular and organic, from different floral sources. The results showed the presence of fipronil desulfinyl, the main degradation product of fipronil, in some samples, even among the organic ones.

Keywords: honey, fipronil, degradation products, gas chromatography, matrix matched method

INTRODUCTION

Every day, honeybees (*Apis mellifera*) make successive flights, covering a wide area, collecting nectar, water and pollen from flowers. During travel, they come into contact with various particles (e.g. pesticides) and microorganisms in the air, soil or water that may become embedded on the surface of their bodies or be inhaled and adhered to their respiratory system.

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Bees and their products can be considered as bioindicators of environmental contamination.[1] The level of contamination of hives by pesticides is directly related to the proximity of a source of pollution and thus can provide information about a specific polluted environment.[2-4] Although honeybees are not targeted by pesticides, their exposure to these products can cause their death in cases of acute toxicity,[3] or impair their foraging behavior and affect colony health and development in cases of chronic toxicity.[4]

The pesticides, when carried to the hive, contaminate the bee products (honey and pollen), reducing their beneficial properties. These products, if highly contaminated, can pose threats to human health when ingested.[5] In addition to foraging contamination, bees and honey can also be contaminated by direct application of pesticides to treat hives.[6]

The loss of hives, a phenomenon known as colony collapse disorder, observed in several countries in the northern hemisphere,[7,8] has been highly associated with diseases caused by Varroa (an ectoparasite mite), Nosema (a kind of fungus), certain viruses, and exposure to pesticides.[9] Thus, concern for the preservation of bees is increasing worldwide, especially since about 80% of plant species depend on pollination to exist, and bees are important pollinators.[10] Therefore, the loss of bees can cause a collapse in the economy of many countries, both by reducing the productivity of honey derivate and negatively impacting agriculture due to decrease in pollination.[7,8,10]

Fipronil, 5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile, is a widely used insecticide that belongs to the chemical family of phenylpyrazole.[11] This compound interferes with Gamma-Amino Butyric Acid (GABA) receptors, interrupting the influx of nerve transmissions. In sufficient doses, it causes excessive neural excitation, severe paralysis, and insect death. The main degradation products of fipronil (Figure 1) also showed strong insecticidal properties.[11,12]

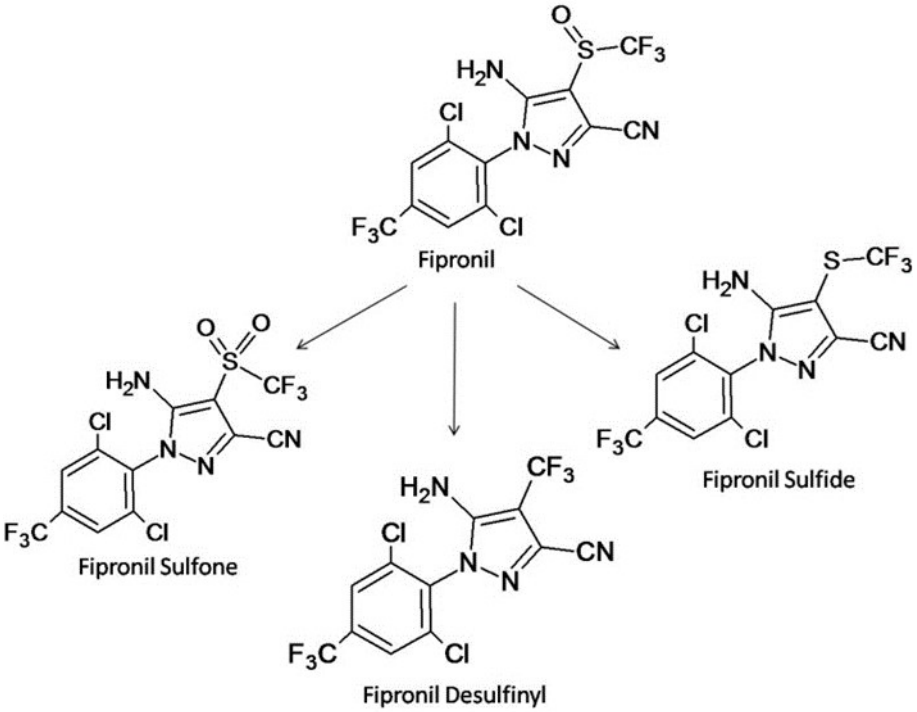

Figure 1. Fipronil and its degradation products: fipronil sulfone, fipronil desulfinyl and fipronil sulfide.

Fipronil has been extensively studied in recent years due to its harmful effects on non-target organisms, such as birds, aquatic organisms and honeybees.[12] For a variety of birds, this compound is highly toxic

through acute and chronic exposure. For aquatic organisms, it can be accumulated in tissues. For honeybees, fipronil has high acute toxicity, and its lethal dose (LD50) ranges from 4 to 6.2 ng/bee.[13,14] Fipronil degradation products, even at low concentrations, have also shown high toxicity to aquatic organisms.[11]

A relevant study confirmed the threat of fipronil to *Apis mellifera* and found that sublethal exposure of fipronil causes motor and behavioral changes in bees, culminating in the collapse of all colonies and the abandonment of hives. These researchers strongly recommended discontinuing the use of this active ingredient.[15]

Several works in the literature describe the determination of fipronil residues in several environmental samples[16] and different honey matrices.[17-21] However, to the best of our knowledge, there is no scientific paper dedicated to optimizing the figures of merit (e.g., low LOQ and high precision) for simultaneous quantification of fipronil (F) and its three most important degradation products, namely fipronil desulfinyl (FD), fipronil sulfone (FSO) and fipronil sulfide (FSE) in commercial honey samples reported in the literature. Therefore, the objective of this study was the development and validation of a fast and accurate method using SPE and GC-ECD for the determination of F and its most frequent degradation products in commercial honey samples.

MATERIALS AND METHODS

Chemicals and materials

Certified individual standards of F (97%), FSO (100%), FD (98%), FSE (99.6%) and the internal standard (IS) decachlorobiphenyl (PCB209) were purchased from AccuStandard (New Haven, USA). Residue analysis-grade methanol, n-hexane, isopropyl alcohol and acetone were purchased from Tedia (Ohio, USA) and ultrapure water was obtained from a Milli-Q® system (Millipore, Milford, MA, USA). The stock and working solutions of each compound were prepared in methanol and stored at 4 °C. Solid phase cartridge, C18, was purchased from Silicycle (Specifications: C18-17%; Bed Weight - 500 mg; Cartridge Volume - 6 mL; Ultrapure Silica Gels). For extraction under vacuum, an SPE manifold (Supelco, Sigma Aldrich, USA) coupled to a vacuum pump (Alfa Mare, Brazil) was used. For method optimization and validation, an uncontaminated honey sample was used as a blank sample for matrix-matched standards.

Sampling

Twenty-eight samples of commercial honey were purchased in local markets in Brasilia (Brazil). These samples come from different floral sources, being twelve predominantly wild, two from orange tree, one from eucalyptus and the other thirteen samples do not present this information. All samples were kept in their original packaging at room temperature in a dark place. Nine of these samples were organic honeys.

Extraction

The extraction method was enhanced by optimizing it, leveraging insights from prior research as a foundation.[18] Initially, the C18 solid phase cartridges (500 mg / 6 mL) were preconditioned by passing 5 mL of methanol followed by 5 mL of purified water (both by gravity) and not allowing complete drying of the sorbent after each wash. An aqueous solution (15 mL) was prepared with 5 g of uncontaminated honey, spiked with 400 µL of F standard and extracted.

Four trials were done to evaluate and select the solvent extraction mixture to be used. They were performed in duplicate with the following solvent mixtures and under vacuum: (1) 3 x 3 mL of methanol, (2) 3 x 3 mL of isopropyl alcohol, (3) 2 x 3 mL of methanol + 3 mL of isopropyl alcohol and (4) 3 x 3 mL of a mixture of n-hexane:isopropyl alcohol (1:1, v/v). Solvent extraction was performed at concentration levels of approximately 0.19 µg g^{-1}. This step is presented in Figure 2A.

Each honey sample (1.0 g) was diluted with water to a volume of 3 mL and a 1 mL aliquot of this solution (previously evaluated to avoid saturation of the cartridge) was passed through the pre-conditioned solid phase cartridge at a flow-rate of about 10 mL min^{-1}. Thereafter, the SPE was washed with 5 mL of water and

the retained compounds were finally eluted by passing 3 x 3 mL of methanol. The eluate was evaporated to dryness using a gentle steam of nitrogen at 40 °C, resolubilized in methanol containing 0.032 μg mL^{-1} of the IS and quantitatively transferred to a 1-mL vial. For analysis, 1 μL of each extract was injected into the GC-ECD system. This step is presented in Figure 2B

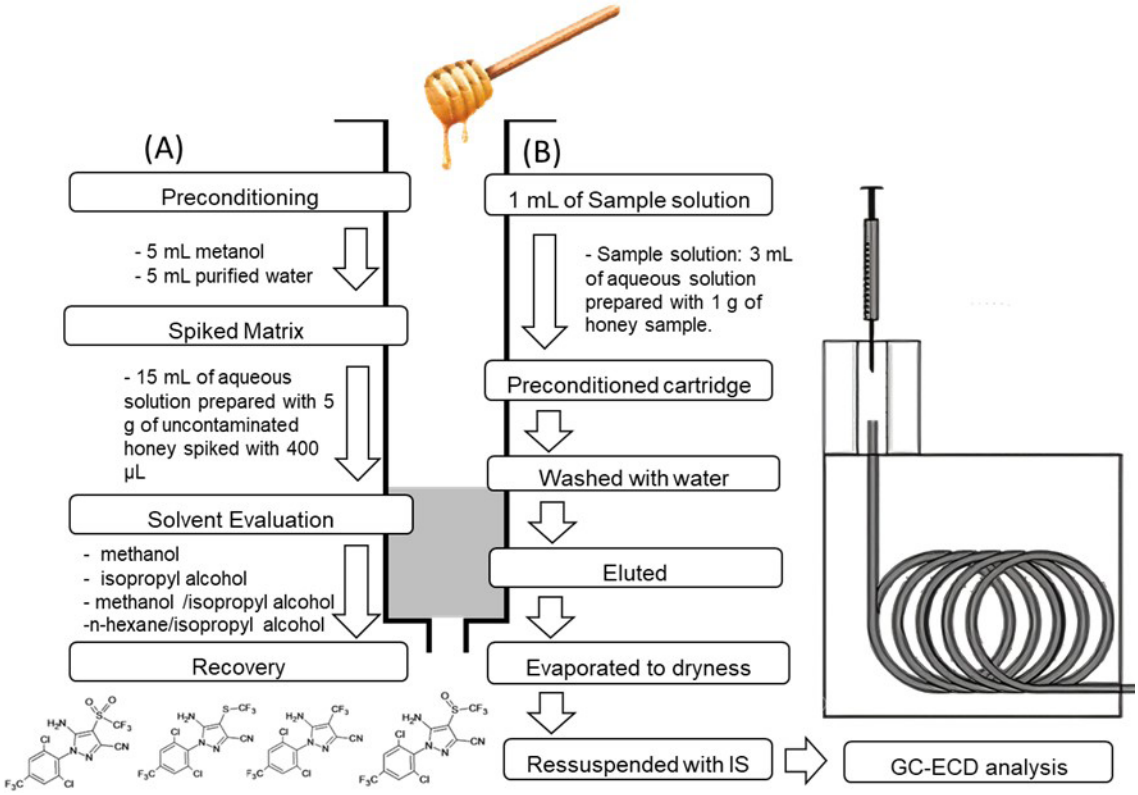

Figure 2. Sample preparation scheme. (A) strategy sequence for solvent selection and (B) sample preparation.

GC-ECD conditions

The chromatographic conditions were initially based on previous work.[22] A Shimadzu GC-2010 gas chromatograph equipped with an electron capture detector (GC-ECD), with a 30 m × 0.25 mm i.d. capillary column coated with a 0.25 μm thick film of 5% phenyl and 95% methylpolysiloxane (Rtx®-5 Restek) was used. Helium was used as carrier gas at a flow-rate of 1 mL min^{-1}. The injection port temperature was 260 °C and 1 μL of standard and samples were injected under splitless mode for 2 min, followed by the split ratio of 1:20. The detector temperature was 305 °C, with nitrogen as make up gas (20 mL min^{-1}). The oven temperature program was: 100 °C (1 min), 15 °C min^{-1} up to 230 °C, 2 °C min^{-1} up to 256 °C (2 min) and 20 °C min^{-1} up to 280 °C (10 min). The total analysis time was 35.87 min.

Analytical Performance and Method Validation

Due to low linearity observed in the analytical curves in methanol, additional curves were prepared in honey extract free of the target compounds (blank honey extract). IS was used in all curves. The comparison of the analytical curves (in methanol and honey extract) for each analyte was based on their angular coefficients. All analytical curves are presented in Table I, each with 6 levels called P1, P2, P3, P4, P5 and P6 in triplicates, and matrix-matched standards were produced according to described previously in the Extraction topic. Pesticides stock and working solutions were prepared in methanol.

Table I. Concentration of F, FD, FSO and FSE in methanol and in honey extracts for each point of the calibration curve

Analyte	Analytical Curve (μg mL^{-1})					
	P1	P2	P3	P4	P5	P6
F	0.0230	0.0322	0.0414	0.0552	0.0690	0.0828
FD	0.0240	0.0336	0.0432	0.0576	0.0720	0.0864
FSO	0.0236	0.0330	0.0425	0.0566	0.0708	0.0850
FSE	0.0230	0.0322	0.0414	0.0552	0.0690	0.0828
F	0.0230	0.0322	0.0414	0.0552	0.0690	0.0828
FD	0.0240	0.0336	0.0432	0.0576	0.0720	0.0864

A validation of the method was carried out with the following parameters: matrix effect, analytical curve and linearity, limits of detection (LOD) and limits of quantification (LOQ), precision (repeatability and intermediate precision) and recovery.

Linearity was evaluated according to the coefficient of determination (r^2) of all analytical curves with a minimum acceptable correlation coefficient equal to 0.99.[23] LOD was established considering the lowest concentration capable of generating a detectable signal, while LOQ was the lowest concentration capable of generating a linear analytical curve (P1 in Table I). For recovery assays with an acceptance criteria between 70 and 120%[24] a honey sample free of the target compounds was fortified at two levels, 100 and 200 ng g^{-1}, for each compound. The precision of the method, in terms of repeatability, was assessed by extracting and analyzing fortified samples at 0.046 μg mL^{-1} in six replicates. To evaluate the intermediate precision of the method, different days were used, also in six replicates. The acceptance criteria was ≤ 20%[24] and precision was calculated according to the Equations 1 and 2, as follows:

$$s = \sqrt{\frac{\sum_{i=1}^{I}\sum_{j=1}^{L}(\hat{x}_{ij}-\bar{x}_i)^2}{I\,(J-1)}} \qquad (1)$$

Where, S is the combined standard deviation, I is the number of samples and J is the number of replicates.

Precision was assessed using the relative standard deviation (RSD) in accordance to equation 2:

$$RSD(\%) = \frac{s}{\bar{x}}\,100\% \qquad (2)$$

RESULTS AND DISCUSSION

Solvent selection

In order to evaluate solvents with a wide range of polarity for elution, different solvents and mixtures were tested. The results of the recovery studies are shown in Table II.

Performing a F-test to compare the recovery results, it can be seen that the recovery for the four solvent combinations did not differ statistically, as any calculated F-value was greater than the unilateral limit of the F-distribution of Fisher-Snedecor with 5% significance level to 3 and 6 degrees of freedom (F_{critic} = 4.191). Thus, pure methanol was chosen as the elution solvent, considering the availability of methanol in the laboratory and the convenience of using the same solvent for extraction and instrumental analysis.

Table II. Recoveries for the tests of the choice of solvent for F, FD, FSO and FSE with the respective calculated values for the F-test

Analyte	Hexane/Isopropyl alcohol (%)		Isopropyl alcohol (%)		Methanol/Isopropyl alcohol (%)		Methanol (%)		F-test
	R1	R2	R1	R2	R1	R2	R1	R2	
FD	85	98	85	100	101	99	97	84	0.55
F	65	70	59	51	73	69	73	60	2.87
FSO	50	54	45	49	55	52	55	50	1.82
FSE	71	73	70	72	74	72	71	72	1.11

Degradation of the compounds

F and its degradation products apparently degraded during storage or during chromatographic analysis. Confirmation of the presence of degradation products was performed by injecting each pesticide standard solution individually. In the FD, F and FSO chromatograms more than one peak was observed. In the chromatogram of the combined standard solution (Figure 3), additional peaks (e.g., F+FSO and two FD peaks) can be seen.

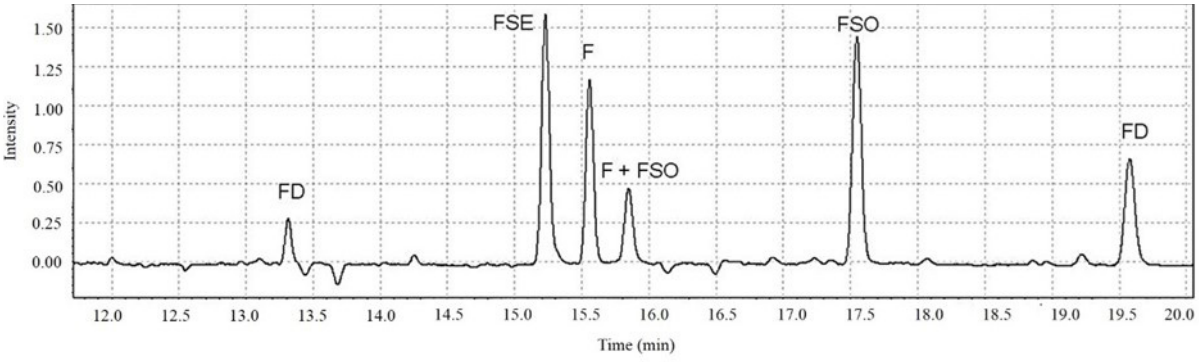

Figure 3. GC-ECD chromatogram for the standard solution containing F, FD, FSE and FSO.

Validation conditions

The analytical curves in methanol did not show good linearity for all analytes in question, with a determination coefficient lower than 0.990 for F, FSO and FD. The first alternative to improve the linearity of the analytical curves was to build a new curve considering the sum of the area of all analyte signals (Σ F, FSO, FD and FSE) *versus* the sum of their concentrations. The new equation showed better linearity (r^2 = 0.999) compared to the individual curves, but lost information about the individual analytes.

Thus, in order to obtain better results for each individual analyte, new standard solutions were prepared in blank honey extracts (matrix-matched). Comparing each analytical curve in methanol with each analytical curve in blank honey extract, a strong matrix effect can be observed, resulting in better sensitivity and linearity for the matrix-matched curves, as can be seen in Figure 4.

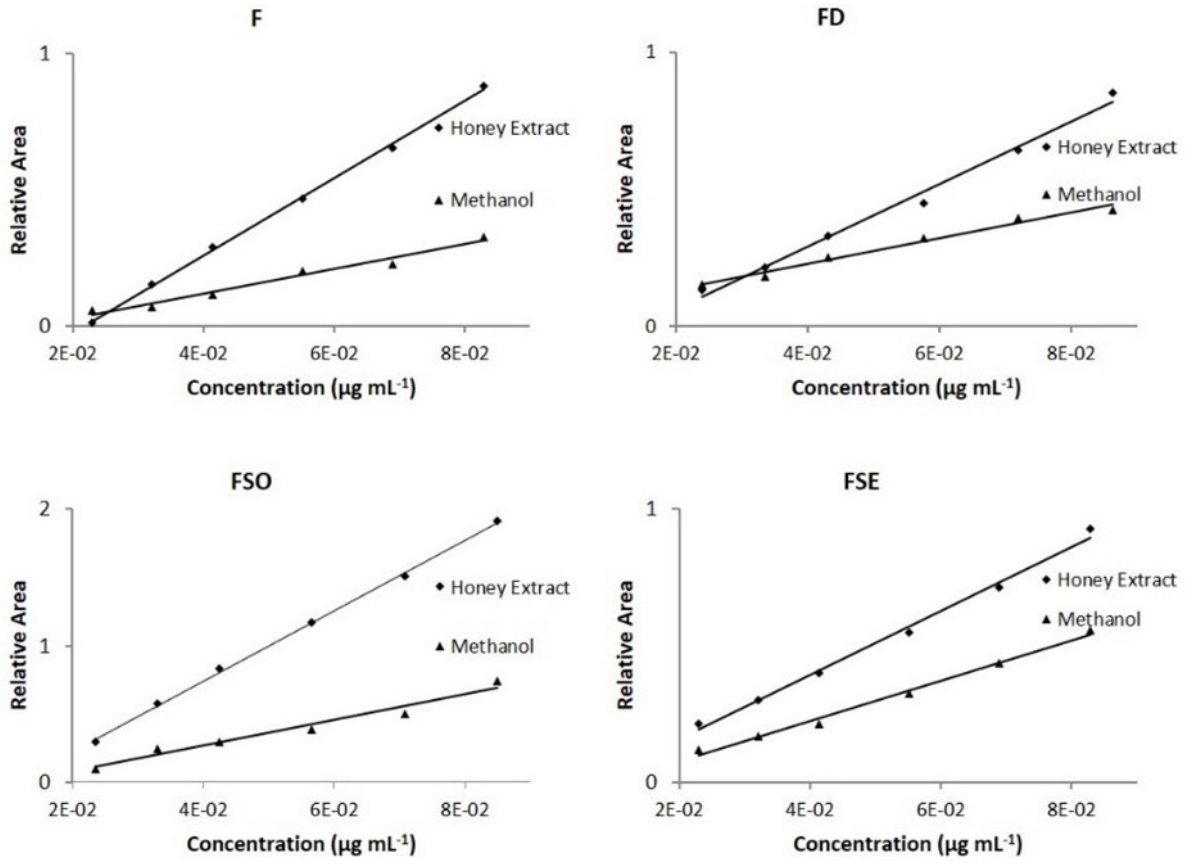

Figure 4. Analytical curves of F, FD, FSO and FSE in methanol and in honey blank extracts with PCB 209 as IS.

This matrix effect may be due to the injector, low analyte concentrations and matrix properties. In relation to the injector, when standards are prepared in pure solvent and analyzed by GC, the active sites of the liner formed by free silanol groups and metal are available for the retention of the analytes, causing a lower transfer of the molecules to the column and consequently to the detector. When standard solutions prepared in blank honey extracts are injected, a competition between the matrix compounds and the analytes for the active sites of the liner occurs, allowing a greater amount of the pesticide to reach the detector.[25]

A stronger matrix effect was also observed at lower analyte concentrations, probably because if present at higher concentrations, the percentage of analyte that is trapped in the active sites of the liner will be much lower and the error associated with each injection will also be less expressive.[25] With regard to matrix properties, the sample and co-extractives can also influence the strength of the matrix effect, depending on the size of molecules, thermal stability, polarity and volatility. Co-extractives such as lipids, pigments and other high molecular weight compounds present in honey can remain in the extract, interfering with chromatographic analyzes and promoting a matrix effect.[25]

For all the above reasons the calibration was performed using matrix-matched standards prepared as described in the experimental section.

The parameters for linear regression (y = ax + b) obtained for a six-level calibration curve, in triplicates, are shown in Table III. Good linearity was obtained for all analytes in the concentration range within the evaluated interval, with determination coefficients greater than 0.990.

Table III. Matrix-matched analytical curve parameters and limits of the method for F and its degradation products

Analyte	Linear regression y = ax + b	r^2	Limits of the method (μg g^{-1} of honey)	
			LOD	LOQ
F	y = 14.17x - 0.108	0.998	0.0138	0.069
FD	y = 11.39x - 0.165	0.990	0.0101	0.072
FSO	y = 25.85x - 0.093	0.998	0.0097	0.069
FSE	y = 11.77x + 0.020	0.993	0.0091	0.071

LODs and LOQs

The LODs were established considering the lowest concentration capable of generating a detectable signal, and the LOQs were established as the lowest concentration capable of composing a linear analytical curve.[23] The LODs and LOQs for F and its degradation products are also presented in Table III, expressed per mass of analyte per mass of honey. These values are similar to those obtained by Flores-Ramirez et al. when working with the same analytes in soil samples.[22]

The LODs reported in the literature for quantification of F and its degradation products in honey through a multicomponent method vary from 0.83 to 1.16 ng g^{-1} of sample using solid phase extraction and LC/MS-MS, while the values found in this work vary between 9.10 and 13.8 ng g^{-1}. Despite achieving lower LODs, techniques such as LC/MS-MS[26] are more sophisticated and expensive compared to GC-ECD, which allows performing analyzes with good sensitivity and linearity. A more recent study compares the determination of F and some of its degradation products in chicken eggs, using LC-MS/MS and GC-ECD techniques. The work concludes that the methods for the analyzed samples are extremely compatible, presenting practically equal results.[27]

Precision and Recovery

Method precision was determined by repeatability and intermediate precision, expressed by relative standard deviations, RSD_R and RSD_{IP}, respectively. These data are shown in Table IV. The method was found to be precise (RSD < 10%) for all the compounds studied at both spiking levels.

Table IV. Precision (n = 6) in terms of repeatability (RSD_R) and intermediate precision (RSD_{IP}) and recovery for F and its degradation products

Analyte	Precision		Recovery (%)	
	RSD_R (%)	RSD_{IP} (%)	Spike level 100 ng g^{-1}	Spike level 200 ng g^{-1}
F	6	10	99 ± 6	74 ± 4
FD	4	8	71 ± 3	72 ± 5
FSO	3	7	84 ± 7	70 ± 6
FSE	5	8	79 ± 4	71 ± 2

The recovery results of the honey samples, at two levels of fortification, were between 70 and 99%, as shown in Table IV. Considering the acceptability criteria for recoveries between 70 and 120%,[24] it can be concluded that the method is in agreement with the required parameters.

Prior research on fipronil and its degradation products in honey

While there are some review papers on the determination of pesticides in honey or other food samples,[28-32] there is none exclusively dedicated to F and its degradation products. Even though there is a growing interest in multi-analyte methods, the determination of F and its derivatives in honey is not yet well-established in the literature. Thus, the quest for creating a robust method remains a challenge, primarily due to the risk that F and its derivatives pose to bees.

Table V presents some analytical strategies previously used to determine F and some of its degradation products in honey samples. As we can see, some more sophisticated methods require a more complex sample preparation, such as the use of extraction and clean-up steps. The addition of more steps, combined with more expensive analytical methods, makes the method even more costly and, consequently, may hinder the analysis of such importance in areas of interest.

Even the more sophisticated methods in the literature, such as LC-MS/MS, have some merit figures similar to those found in this study, such as recovery values, linear range, and LOD, as presented in Table V. It is also worth highlighting that among the methods found, few focused on the determination of its degradation products, which are important sources of information about the contamination of floral sources for honey production.

Table V. Analytical strategies for the determination of F and some of its degradation products in honey samples reported in the literature

Analytes	Sample Preparation		Analitycal Method	Linear range	LOD	Recovery (%)	Ref
	Extraction	Clean-up					
F	LLE[a]	SPE by Florisil Columm	GC-ECD	0.001 – 2 mg kg^{-1}	1 µg kg^{-1}	72.0 – 93.0	17
F	Modified QuEChERS[b]		LC-UV	0.03 – 0.25 mg kg^{-1}	30 µg kg^{-1}	70.7 – 101.1	33
F	OCLLE[c]		LC-MS/MS	2.5 – 10 ng g^{-1}	0.015 µg kg^{-1}	72.0	34
F	Modified QuEChERS		LC-MS/MS	0.005 – 0.05 mg kg^{-1}	0.0004 mg kg^{-1}	68.4 – 83.8	35
F		SPE	GC-MS	10 – 300 ng g^{-1}	1.4 ng g^{-1}	99.0 – 106.0	36
F	LPE[d]	SPE by Florisil	UHPLC-MS/MS	0.1 – 5 µg kg^{-1}	0.03 -1.51 µg kg^{-1}	75.0 -125.0	37
F	QuEChERS		LC-MS/MS		0.01 mg kg^{-1}	87.6 – 111.0	5
F	LPE		UHPLC-MS/MS		0.05 – 10 µg kg^{-1}	81.9 – 98.1	38
F	QuEChERS		LC-MS/MS	0.2 – 10 ng g^{-1}		30.0 – 96.0	39

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Table V. Analytical strategies for the determination of F and some of its degradation products in honey samples reported in the literature (continuation)

Analytes	Sample Preparation		Analitycal Method	Linear range	LOD	Recovery (%)	Ref
	Extraction	Clean-up					
F, FSO, FSE, FD, and fipronil carboxamide	LPE	SPE by Florisil Adsorbent	LC-MS/MS	2 – 18 ng mL^{-1}	0.83 – 1.16 ng g^{-1}	89.9 – 98.8	26
F, and FSO	QuEChERS		LC-MS/MS	1 – 100 ng g^{-1}	1.3 and 0.3 ng g^{-1}	82.0 – 97.0	40
F, FD, FSE, and FSO	QuEChERS		LC- MS/MS	0.001 – 0.1 mg kg^{-1}	0.001 mg kg^{-1}	75.0 – 120.0	41
F, FD, FSE, and FSO		SPE	GC-ECD	0.0230 – 0.0864 µg mL^{-1}	0.0091 – 0.0138 µg kg^{-1}	70.0 – 99.0	This study

[a]LLE – Liquid-Liquid Extraction; [b]QuEChERS – Quick, Easy, Cheap, Effective, Rugged and Safe; [c]OCLLE – On-Column Liquid-Liquid Extraction; [d]LPE – Liquid-phase

Analysis of commercial honey samples

The only compound found in the analyzed samples was FD. However, in some of these samples, the generated signal was lower than the LOQ. The results are presented in Table VI. No signals related to the retention time of F, FSO and FSE were observed.

Table VI. Results of analyzes of honey samples for FD

Organic samples	Concentration (µg g^{-1})	Conventional Sample	Concentration (µg g^{-1})	Conventional Sample	Concentration (µg g^{-1})
1	<LOQ	11	<LOQ	21	<LOQ
2	<LOQ	12	<LOQ	22	<LOQ
3	0.13 ± 0.01	13	<LOQ	23	0.081 ± 0.003
4	<LOQ	14	0.16 ± 0.03	24	0.075 ± 0.001
5	<LOQ	15	0.094 ± 0.009	25	<LOQ
6	0.10 ± 0.01	16	<LOQ	26	0.129 ± 0.005
7	<LOQ	17	<LOQ	27	0.076 ± 0.004
8	0.079 ± 0.002	18	0.075 ± 0.003	28	<LOQ
9	<LOQ	19	0.080 ± 0.001		
10	<LOD	20	<LOQ		

FD, the main degradation product of F, is very persistent in the environment and considered bioacumulative.[42,43] This compound proved to be more toxic to rats and mosquitoes than F and presented high toxic potential for human health.[44] Therefore, in addition to being a potentially dangerous contaminant, it can also be considered a marker of fipronil use.

The presence of F and its degradation products does not depend on the production type of honey, whether organic or conventional, probably because bees, when foraging, can go further than expected reaching areas of conventional agriculture. Thus, although the beekeeper does not directly use any type of pesticide in the treatment of hives, bees can be contaminated in crops treated with pesticides. Furthermore, in Brazil the use of F has been suspended due to serious adverse effects on bees.[45]

CONCLUSIONS

The method for determining F and its degradation products in honey samples using IS matrix-matched analytical curves proved to be simple and effective. Extraction with a C18 SPE column and elution with methanol resulted in clean extracts capable of being injected in the GC-ECD system.

Validation parameters were all within the expected range, resulting in recoveries ranging from 70 ± 6 to 99 ± 6%. All determination coefficients for the IS matrix-matched analytical curves were above 0.990, higher than the ones obtained for external standard analytical curves in methanol.

The optimized method was applied to 28 commercial honey samples, including organic and conventional, and 11 samples showed a signal of FD higher than LOQ, regardless of whether the sample was organic or not.

In this study, we cannot infer the initial contamination level of each sample, as these active ingredients are subject to degradation over time. Thus, it is possible that samples with pesticide concentration results lower than the LODs were contaminated at the time of collection, but with the passage of time the analytes were completely degraded.

The analyte found in the samples, FD, is a product of the photodegradation of F and represents a potential risk to human and bee health.

Conflicts of interest

The authors declare that they have no financial conflicts of interest.

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