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Detection of Fluvalinate Residues In Beeswax and Honey using High Performance Liquid Chromatography with Diode Array Detector

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Abstract

Fluvalinate is a synthetic pyrethroid widely used to control honey bee infestations caused by *Varroa destructor* and *Acarapis woodi* mites. Contamination of bee products with fluvalinate residues may pose a serious health risk to consumers and is therefore a major concern. HPLC with diode array detector (HPLC-DAD) method was developed for identification and quantification this acaricide in beeswax and honey. The method involves a modified, easy-to-perform liquid-liquid extraction of fluvalinate. As a result of selecting the optimal extracting solvent, it was found that the best extraction of the acaricide was achieved using a mixture of dichloromethane:isooctane solvents

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in a ratio of 2:8 (v/v). The validation characteristics of the method were satisfactory. The specificity and linearity of the method were proven. The fluvalinate recovery from beeswax and honey matrix was 85.3-96.4% and 88.7-98.9%, respectively. The limit of detection (LOD) for recovered fluvalinate from beeswax was 0.02 µg/g, from honey – 0.005 µg/g, and the limit of quantification (LOQ) was 0.05 and 0.012 µg/g, respectively. The repeatability and reproducibility of the method, expressed as relative standard deviation (RSD), were less than 5%. Also, using the proposed method, an analysis of the migration and accumulation of fluvalinate in beeswax and honey during and after the treatment of bees with fluvalinate containing strips was carried out. The study was carried out at the experimental apiary located in the Moscow region. The samples of beeswax and honey were collected both during veterinary treatment of bees (for 30 days) and after strips removal for 6 months. The data, obtained for the first time over such a long period of sample analysis, demonstrated the migration of fluvalinate from honey into beeswax and its accumulation in this matrix. The obtained results may be useful for monitoring and controlling the fluvalinate content in beekeeping products.

Introduction

Beeswax and honey are complex biological substances that contain a variety of organic and inorganic components.[1–3] They have been widely used in the food, pharmaceutical, cosmetic, and medical industries due to their unique properties.[3-5] The decline in beekeeping product production is caused by the impact of dangerous invasive diseases on honey bees (*Apis mellifera*), such as those caused by *Varroa destructor* and *Acarapis woodi* mites.[6–8]

The *Varroa* mite is a parasite that infests adult bees and can also infect open and sealed brood.[8] During the active period of a bee colony, the sealed brood may contain between 70 and 90% of these mites. The mites penetrate deep into the abdominal segments of bees, feeding on their hemolymph.[9] In 2019, Ramsey S.D. found that the mite *Varroa destructor* also feeds on the fat body of hive bees.[10] The danger of bee colonies being affected by Varroatosis is aggravated by the fact that *Varroa destructor* is a carrier of dangerous viral infections of honey bees.[11,12] As a result, Varroatosis leads to the weakening of bee colonies, and in the case of severe mite infestation, to their death.[13]

Acarapis woodi mites infect the first thoracic tracheae of bees, settling in them and penetrating the abdominal and head air sacs. A single bee may contain up to 150 individuals of these mites. Adult workers, queens, and drones can be affected. With acarapidosis, the body of the bee loses hemolymph and protein, due to the disruption of tracheal walls and oxygen starvation. When bees are infected by *Acarapis* mites, flight muscle degeneration occurs, glycogen levels decrease, and cellular mitochondria change. This leads to a reduction in the lifespan of affected bees, decreased resistance to other pathogens, and an increased risk of septic diseases.[14–16]

It is worth noting that a complex epizootic situation with regard to the proliferation of invasive bee diseases is currently being observed in apiaries across numerous countries. Varroatosis and acarapidosis pose a significant challenge to beekeeping operations on a global scale.[13, 17–19]

The most commonly used chemical compounds with acaricidal action against the above mentioned mites include synthetic pyrethroids, in particular fluvalinate, which has been used for this purpose since the 1990s.[20] Fluvalinate ((RS)-α-cyano-3-phenoxybenzyl N-(2-chloro-α,α,α-trifluoro-p-tolyl)-D-valinate) is a lipophilic, non-volatile compound synthesized from racemic valine.[21] Its mechanism of action is based on blocking the transmission of nerve

impulses, which, as a result, causes a violation of coordination of movements, paralysis and death of mites. This compound serves as an active ingredient in various drugs such as Apistan®, Manhao Pro®, Mavrik®, Klartan®, etc.[22–24]

The contamination of bee products with pyrethroid residues poses a significant health hazard to consumers, as evidenced by a number of studies.[2,25–28] Specifically, when fluvalinate-based medications are used, their residues have been detected in beeswax and honey at micro- and nanoscale concentrations.[2,5,28] The European Medicines Agency has not yet established a maximum permissible limit (MRL) for the content of fluvalinate in bee products.[23] In accordance with European (EU) regulations, the maximum residue limits (MRLs) for fluvalinate in honey should not exceed 0.05, 0.02, and 0.01 mg/kg, respectively, in Europe, the United States, and Australia.[29] Switzerland has also set a standard of 0.05 mg/kg for fluvalinate in honey.[21] According to the Technical Regulations of the Customs Union "On the Safety of Food Products" (TR CU 021/2011), there are no established maximum permissible amounts for fluvalinate. However, in Russia, its presence in products related to beekeeping is not permitted.[30]

The detection of residual amounts of fluvalinate and other acaricides in beeswax and honey is accomplished through the application of chromatographic techniques. Despite the fact that in recent years gas or liquid chromatography in combination with mass spectrometry (GC-MS and LC-MS) and tandem mass spectrometry (GC-MS/MS and LC-MS/MS) have been most often used for this purpose,[1,5,28,31–38] a number of researchers note the advantages of high-performance liquid chromatography (HPLC): high sensitivity, high separation efficiency, and ease of implementation.[21,23,31,39,40]

The aim of this work was to develop a highly sensitive HPLC with a diode array detector (HPLC-DAD) method for detecting residual amounts of fluvalinate in beeswax and honey, including a modified, easy-to-perform extraction of this compound from the above-mentioned biological matrices. Furthermore, we employed this method to investigate the migration and accumulation of fluvalinate within beeswax and honey both during and after treatment with acaricidal strips containing fluvalinate as an active ingredient. Given the widespread use of this acaricide in beekeeping practices, the findings of this research may prove valuable for monitoring and regulating the levels of fluvalinate in bee products.

Materials and Methods

Reagents

The analytical standard of fluvalinate (Fig. 1) with 98.9% purity ($C_{26}H_{22}ClF_3N_2O_3$, Sigma-Aldrich, USA), acetonitrile (ACN) with 99.9% purity (C_2H_3N, Sigma-Aldrich, USA), HPLC grade water (H_2O, Sigma-Aldrich, USA), isooctane with a purity of 99.5% (iso-C_8H_{18}, Merck, Germany), n-hexane with a purity of 98% (n-C_6H_{14}, Supelco, Germany), dichloromethane with a purity of 99.8% (CH_2Cl_2, Sigma-Aldrich, USA), acetone with a purity of 99.5% ($(CH_3)_2CO$, Sigma-Aldrich, USA) were used in this study.

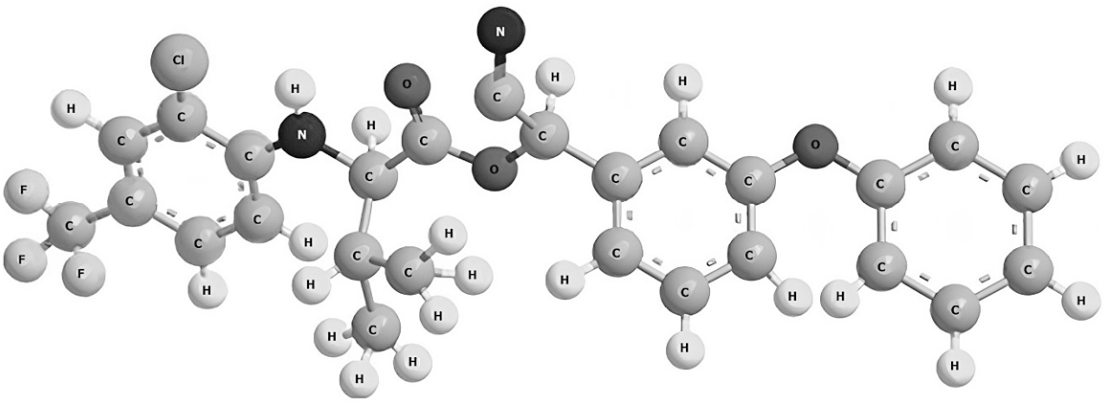

Fig. 1: Fluvalinate 3D structure

Sample Collection

To develop a method for detecting fluvalinate, samples of beeswax (N = 50) and honey (N = 50) collected in May 2023 at the experimental apiary of All-Russian Research Institute of Veterinary Sanitation, Hygiene and Ecology (Moscow Region: 55.71075009980114, 38.097639230600294) were used. In order to study the accumulation and migration of fluvalinate in honey and beeswax of treated bees, bee colonies (N = 30) with a strength of 8-9 bee streets were selected. Each colony received acaricidal treatment using strips containing 40 mg of fluvalinate. These strips were distributed evenly among the hives based on the number of frames. Treatment lasted for 30 days, representing the maximum exposure period for bee colonies to the acaricide. After the treatment period, samples of honey and wax were collected by cutting pieces of honeycomb (3 × 3 cm) from the frames of each hive. The honey was extracted from the combs and the combs were melted down to obtain wax. Samples of honeycomb were collected from both the center and peripheral frames, where acaricidal strips had been placed. The samples were collected at intervals of once every 5 days for 30 days. After 30 days, the acaricidal strips were removed from the hives, but honey and beeswax samples continued to be collected and examined once a month for 6 months. Samples were collected and stored in accordance with the requirements of Interstate Standards 21179-2000 and 19792-2017.[41,42]

Beeswax Fortification

Prior to the addition of fluvalinate, samples of beeswax were subjected to freezing at a temperature of -18 °C, followed by grinding in a mortar. One gram of each sample was then transferred into heat-resistant laboratory beakers, which were subsequently heated to a temperature of 70 °C until the beeswax fully melted. Subsequently, 100 µL of fluvalinate solutions with concentrations ranging from 2000 to 0.244 µg/mL, prepared by two-fold dilution in acetonitrile (ACN), were added to each sample. The specimens were subsequently allowed to cool down to room temperature.

Honey Fortification

1 g of honey was dissolved in 10 mL of distilled water. Next, 1 mL of fluvalinate solution in ACN was added at concentrations from 200 to 0.0061 µg/mL, prepared in ACN by two-fold dilution.

Method for the Fluvalinate Detection in Beeswax and Honey

The method for fluvalinate determination included the following steps: modified liquid–liquid extraction (LLE)[22,43] and detection by HPLC-DAD.

Fluvalinate Extraction from Beeswax Matrix

A sample was placed in a vial and heated to 60 °C. In parallel, the solvent was heated in a heat-resistant beaker to the same temperature. The solvent was added to the melted sample. Next, the sample was vortexed until completely dissolved. The solution was transferred to a centrifuge tube and cooled for 30 min at -18 °C. After this, centrifugation was carried out for 10 min at 5000 rpm. The supernatant was poured into a wide-necked beaker, and heated solvent was added to the sediment. The sediment was resuspended using a glass rod, then cooled for 30 min at -18 °C and centrifuged for 10 min at 5000 rpm. This procedure was repeated twice, after which the combined supernatants were allowed to evaporate completely in a fume hood. The extracted material was then dissolved in a volume of 4 mL of ACN. Finally, the resulting solution was filtered through a PTFE (poly(tetrafluoroethylene) membrane with a pore size of 0.22 µm.

Fluvalinate Extraction from Honey Matrix

A solvent was added to the honey solution and the mixture was vortexed. The solution was then left to settle, and the top layer was carefully transferred to a glass container with a wide-mouth. This process was repeated three times, after which the glasses were placed in a fume hood to allow the solvent to completely evaporate. Next, the extracted material was dissolved in 4 mL of ACN (acetonitrile). The resulting solution was filtered through a PTFE membrane with a pore size of 0.22 µm.

Reversed Phase HPLC-DAD

The analysis was performed using Shimadzu LC-20 Prominence chromatograph with diode array detector (Shimadzu, Japan), Shim-pack GWS C18 column (150 × 4.6 mm, 5 µm) in isocratic mode at a flow rate of 1.2 mL/min. Mobile phase is ACN:H_2O in a ratio of 80:20 (v/v). The working wavelength – 256 nm and peak retention time – 6.49 min. Temperature of column was maintained at 30 °C and injection volume of 10 µL was used. Labsolution Software (Shimadzu, Japan) is used to manage analytical data. The calibration curve was constructed based

on the analytical standard fluvalinate. To avoid matrix effects, beeswax and honey known to be free of acaricide residues were used in the study.

Method Validation

The validation process was conducted in accordance with the guidelines on bioanalytical method validation issued by the European Medicines Agency (EMA) and the Russian State Pharmacopoeia, version 15.[44,45] These documents provide detailed instructions for the validation of bioanalytical techniques used to measure drug concentrations in various biological matrices. This choice was based on the accuracy and precision of the recommendations provided in these guidelines. The validation criteria employed included specificity, linearity, accuracy, sensitivity, reproducibility, and repeatability.

Statistical Analysis

MS Excel 2010 Software was used for statistical processing of data obtained. All measurements were performed in triplicate. Student's *t*-test (significance level – $p < 0.05$) was used. Results are presented as means (M) and standard errors of the means (±SEM).

Results

Optimization of Fluvalinate Extraction Technique

To select the optimal parameters for fluvalinate extraction from beeswax, samples from 0.1 to 1 g were used. Acetone, ACN, dichloromethane and solvent mixtures: dichloromethane:hexane (2:8, v/v) and dichloromethane:isooctane (2:8, v/v) were used as solvents. The results obtained are presented in Table 1.

Table 1: Fluvalinate extraction from beeswax

Solvent	Analyzed parameter		Average fluvalinate recovery, %
	Sample weight, g	Solvent volume, mL	
Acetone	0.1	5/3/3	-
		10/6/6	-
		15/9/9	-
	0.5	5/3/3	-
		10/6/6	-
		15/9/9	-
	1.0	5/3/3	-
		10/6/6	-
		15/9/9	-
ACN	0.1	5/3/3	7.9
		10/6/6	9.7
		15/9/9	9.5
	0.5	5/3/3	7.6
		10/6/6	9.1
		15/9/9	9.9
	1.0	5/3/3	7.7
		10/6/6	8.6
		15/9/9	9.7
Dichloromethane	0.1	5/3/3	24.7
		10/6/6	26.8
		15/9/9	28.3
	0.5	5/3/3	22.1
		10/6/6	23.3
		15/9/9	24.9
	1.0	5/3/3	19.8
		10/6/6	21.2
		15/9/9	23.5

Dichloromethane:Hexane (2:8)	0.1	5/3/3	68.7
		10/6/6	68.9
		15/9/9	70.2
	0.5	5/3/3	65.2
		10/6/6	66.1
		15/9/9	67.5
	1.0	5/3/3	62.1
		10/6/6	62.7
		15/9/9	63.5
Dichloromethane:Isooctane (2:8)	0.1	5/3/3	95.7
		10/6/6	95.9
		15/9/9	96.1
	0.5	5/3/3	88.1
		10/6/6	88.7
		15/9/9	89.4
	1.0	5/3/3	84.1
		10/6/6	84.8
		15/9/9	86.2

Table 2: Fluvalinate extraction from honey

Solvent	Solvent volume, mL	Average fluvali -nate recovery, %
Dichloromethane	10	85.9
	10/10	88.4
	10/10/10	89.3
Hexane	10	-
	10/10	-
	10/10/10	-
Isooctane	10	-
	10/10	-
	10/10/10	-
Dichloromethane:	10	88.3
Hexane (2:8)	10/10	89.6
	10/10/10	91.1
Dichloromethane:	10	93.7
Isooctane (2:8)	10/10	95.5
	10/10/10	98.6

The employment of acetone and acetonitrile (ACN) yielded unsatisfactory outcomes, attributable to the limited solubility of beeswax in these solvents. Upon cooling, the mixture rapidly solidified when using acetone, rendering further measurements impractical. This observation was also documented in the investigations conducted by Adamczyk S.[23] Dichloromethane (DCM), DCM:hexane, and DCM:isooctane exhibited an inverse correlation between the degree of extraction and the sample weight, as well as a direct correlation with the volumes of the solvent. The level of extraction when employing DCM did not surpass 28.3%, while that achieved with the DCM:hexane blend reached 70.2%. In both scenarios, the sample mass was minimal, while the solvent volume was maximized. The utilization of the DCM:isooctane combination allowed for extraction rates exceeding 95%, resulting in superior outcomes. Consequently, the optimal results were attained with a DCM:isooctane mixture comprising 0.1 grams of sample and 15 mL of solvent, split between 9 mL each of DCM and isooctane. Nonetheless, a threefold reduction in volume (to 5/3/3 mL) yielded comparable results – 96.1% and 95.7%, respectively.

For the extraction of fluvalinate from honey, 1 g samples were dissolved in 10 mL of distilled water. After adding the solvents, vortex mixing was performed. The resulting mixtures were then left at room temperature until the fractions were separated. Table 2 shows results obtained.

Since using hexane did not form a stratifying gel, but isooctane – the separation into fractions was not complete, which made it impossible to correctly measure the degree of recovery, further study of these options was not carried out. When using dichloromethane and its mixtures with hexane and isooctane, a clear separation of the mixture of honey solution and solvent into two layers/fractions occurred, of which the upper one was the solvent with fluvalinate extracted into it. Dichloromethane and its mixtures showed a direct dependence of the degree of extraction on the solvent volume.

Reversed Phase HPLC-DAD Analysis

Linear Relationship

To construct a calibration curve, working solutions with concentrations from 200.0 to 0.003 µg/mL were prepared from the commercial fluvalinate analytical standard (AS). Figure 2 shows the linear regression equation.

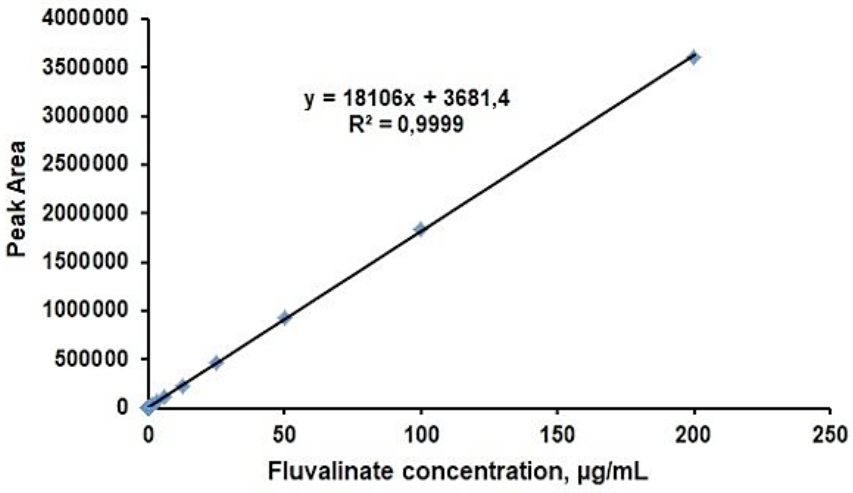

Fig. 2: Fluvalinate analytical standard (AS) calibration curve

From the data presented, it can be seen that the correlation coefficient (R^2) was high and amounted to 0.9999. Therefore, it can be concluded that there is a strong linear dependence of the peak area on the concentration of fluvalinate.

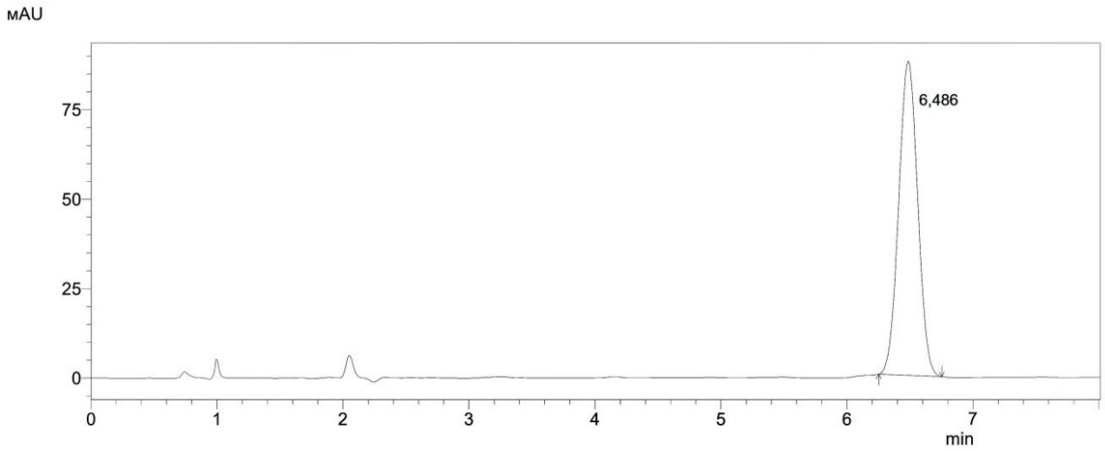

Fig. 3: Chromatogram of the fluvalinate AS (50 µg/mL)

During chromatography, the fluvalinate AS peak had a symmetrical shape, the retention time was 6.49 min, the baseline was stable (Fig. 3). Labsolution Software also generates 3D image of the chromatogram and spectrum profile, which provides more complete analytical information about the substance being studied (Fig. 4).

HPLC of Fluvalinate Extracted from Wax and Honey Samples

The chromatography of the recovered analyte was conducted under the identical conditions as for fluvalinate AS. Figures 5(a) and 5(b) illustrate examples of the obtained chromatograms.

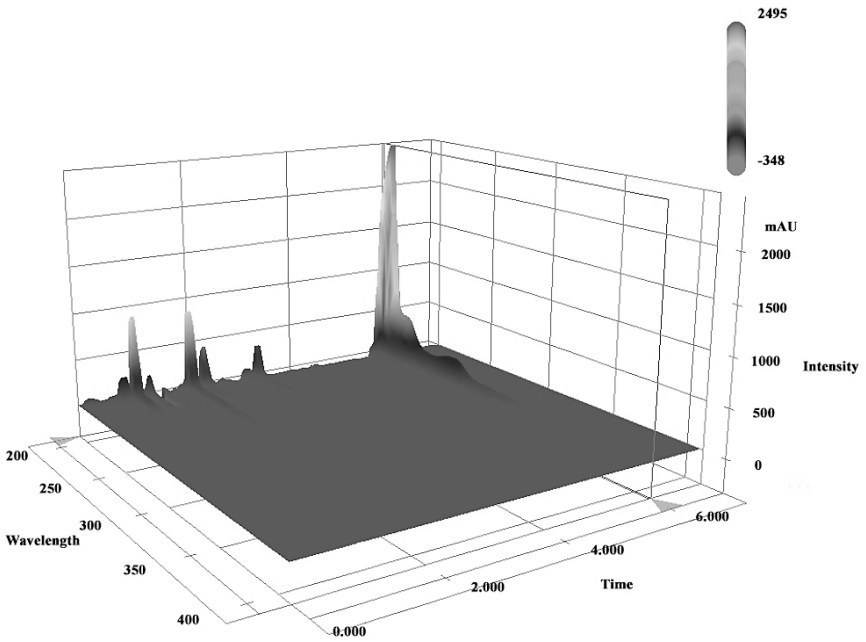

Fig. 4: 3D image of the chromatogram and spectrum of fluvalinate AS

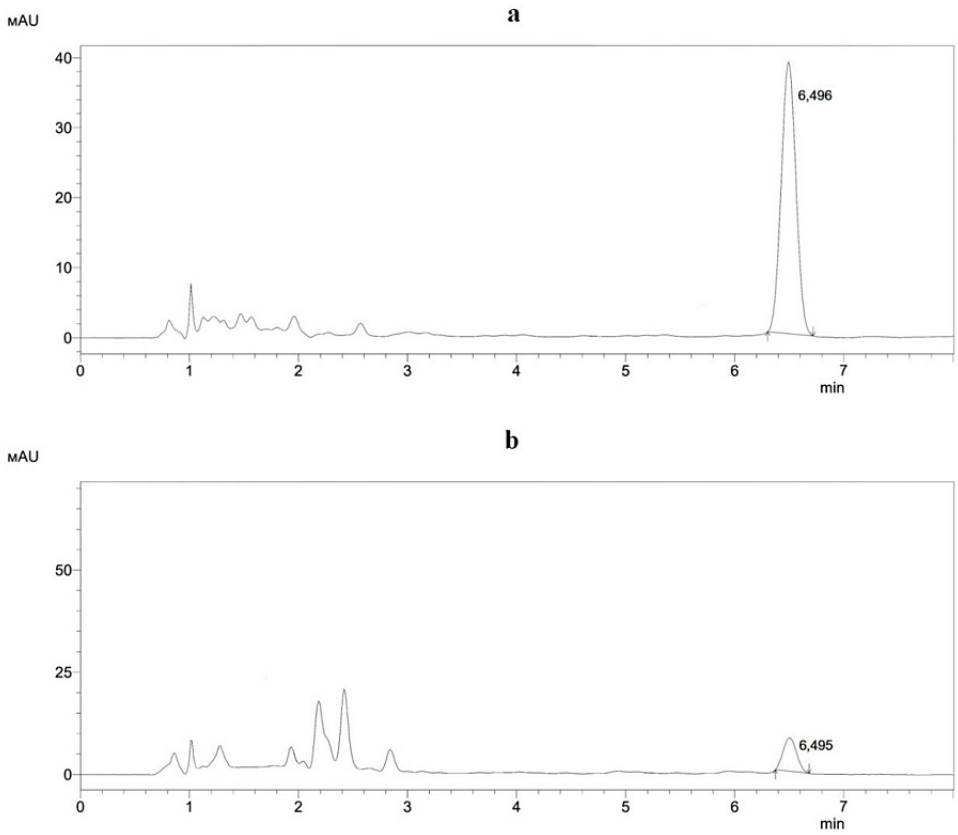

Fig. 5: Chromatograms of fluvalinate recovered from beeswax (a) and honey (b) matrices

As can be seen, the peak retention time coincides with the output of the AS. The peaks are symmetrical and well separated from impurities, allowing for quantitative determination of fluvalinate.

Validation Method Specificity

In order to meet the acceptance criteria, the maximum allowable relative standard deviation of the retention time of the fluvalinate peak in the chromatograms of test samples should not exceed 2.0%. Furthermore, the retention times of the peaks in the chromatograms of both standard and test solutions should differ by no more than 2%. The data obtained is presented in tables 3–5.

Table 3: Relative standard deviation (RSD) of retention times of fluvalinate peaks in standard solution, beeswax and honey extracts

Analysis number	Retention time of fluvalinate peak in standard solution, min			Retention time of fluvalinate peak in beeswax extract, min			Retention time of fluvalinate peak in honey extract, min		
	200 µg/mL	20 µg/mL	2 µg/mL	200 µg/mL	20 µg/mL	2 µg/mL	200 µg/mL	20 µg/mL	2 µg/mL
1	6.486	6.492	6.487	6.496	6.492	6.491	6.495	6.489	6.491
2	6.488	6.491	6.489	6.492	6.494	6.492	6.492	6.488	6.490
3	6.491	6.493	6.488	6.491	6.490	6.489	6.490	6.492	6.488
4	6.493	6.490	6.486	6.493	6.492	6.488	6.491	6.491	6.489
5	6,492	6.489	6.491	6.492	6.494	6.493	6.493	6.495	6.492
6	6.489	6.491	6.492	6.491	6.489	6.491	6.493	6.493	6.496
7	6.487	6.492	6.490	6.494	6.490	6.490	6.488	6.490	6.490
8	6.489	6.490	6.491	6.491	6.492	6.493	6.487	6.490	6.492
9	6.490	6.487	6.488	6.489	6.495	6.492	6.491	6.491	6.494
10	6.491	6.488	6.487	6.493	6.493	6.491	6.490	6.488	6.495
Average value	6.489	6.49	6.489	6.492	6.492	6.491	6.491	6.491	6.492
SD	0.0022	0.0019	0.0023	0.0019	0.0018	0.0016	0.0024	0.0022	0.0026
RSD, %	0.034	0.029	0.031	0.03	0.03	0.025	0.037	0.034	0.04

Table 4: Difference in retention times of fluvalinate peaks in standard solution and beeswax extract

Analysis number	Retention time of fluvalinate peak, min								
	200 µg/mL		Δ t, %	20 µg/mL		Δ t, %	2 µg/mL		Δ t, %
	AS	Beeswax extract		AS	Beeswax extract		AS	Beeswax extract	
1	6.486	6.496	0.154	6.492	6.492	0	6.487	6.491	0.061
2	6.488	6.492	0.061	6.491	6.494	0.0461	6.489	6.492	0.046
3	6.491	6.491	0	6.493	6.490	0.0462	6.488	6.489	0.015
4	6.493	6.493	0	6.490	6.492	0.030	6.486	6.488	0.030
5	6.492	6.492	0	6.489	6.494	0.077	6.491	6.493	0.030
6	6.489	6.491	0.030	6.491	6.489	0.030	6.492	6.491	0.015

7	6.487	6.494	0.107	6.492	6.490	0.030	6.490	6.490	0
8	6.489	6.491	0.030	6.490	6.492	0.030	6.491	6.493	0.030
9	6.490	6.489	0.015	6.487	6.495	0.123	6.488	6.492	0.061
10	6.491	6.493	0.030	6.488	6.493	0.077	6.487	6.491	0.061
Average value	6.489	6.492	0.0427	6.49	6.492	0.0489	6.489	6.491	0.0349

Table 5: Difference in retention times of fluvalinate peaks in standard solution and honey extract

Analysis number	Retention time of fluvalinate peak, min								
	200 µg/mL		∆ t, %	20 µg/mL		∆ t, %	2 µg/mL		∆ t, %
	AS	Honey extract		AS	Honey extract		AS	Honey extract	
1	6.486	6.495	0.1385	6.492	6.489	0.0462	6.487	6.491	0.0616
2	6.488	6.492	0.0616	6.491	6.488	0.0462	6.489	6.490	0.0154
3	6.491	6.490	0.0154	6.493	6.492	0.0154	6.488	6.488	0
4	6.493	6.491	0.0308	6.490	6.491	0.0154	6.486	6.489	0.0462
5	6.492	6.493	0.0154	6.489	6.495	0.0923	6.491	6.492	0.0154
6	6.489	6.493	0.0616	6.491	6.493	0.0308	6.492	6.496	0.0616
7	6.487	6.488	0.0154	6.492	6.490	0.0308	6.490	6.490	0
8	6.489	6.487	0.0308	6.490	6.490	0	6.491	6.492	0.0154
9	6.490	6.491	0.0154	6.487	6.491	0.0616	6.488	6.494	0.0923
10	6.491	6.490	0.0154	6.488	6.488	0	6.487	6.495	0.1231
Average value	6.489	6.491	0.4004	6.49	6.491	0.3387	6.489	6.492	0.4310

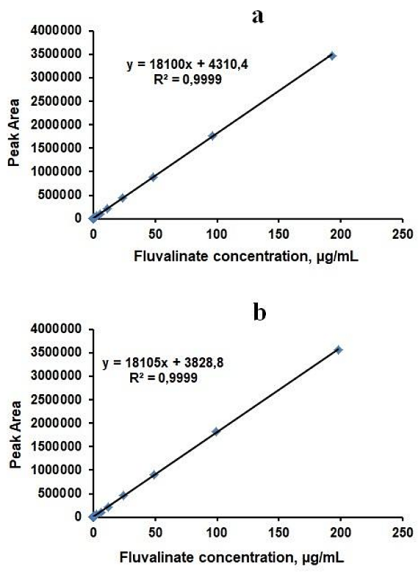

Fig. 6: Calibration curves of fluvalinate recovered from beeswax (a) and honey (b) matrices

The obtained results confirm that all acceptance criteria have been met.

Linearity

Calibration curves were constructed using blank (known to be free of fluvalinate) wax and honey matrices contaminated with different concentrations of the acaricide (Fig. 6 a, b). This calibration procedure avoids the matrix effect. The concentration range was selected in accordance with the sensitivity of the method.

Acceptance criteria – the correlation coefficient (R^2) when checking the linearity of the "peak area – concentration" relationship should be at least 0.9995; the dependence of the peak area on the concentration of the test solution should be described by a linear equation: y = bx+a. As you can see, both criteria were met.

Recovery

To perform the recovery test, empty beeswax and honey matrices were spiked with different concentrations of fluvalinate. The obtained recovery values from the beeswax matrix varied from 85.3 to 96.4%, from the honey matrix – from 88.7 to 98.9% (Fig. 7). The relative standard deviation (RSD) did not exceed 5%.

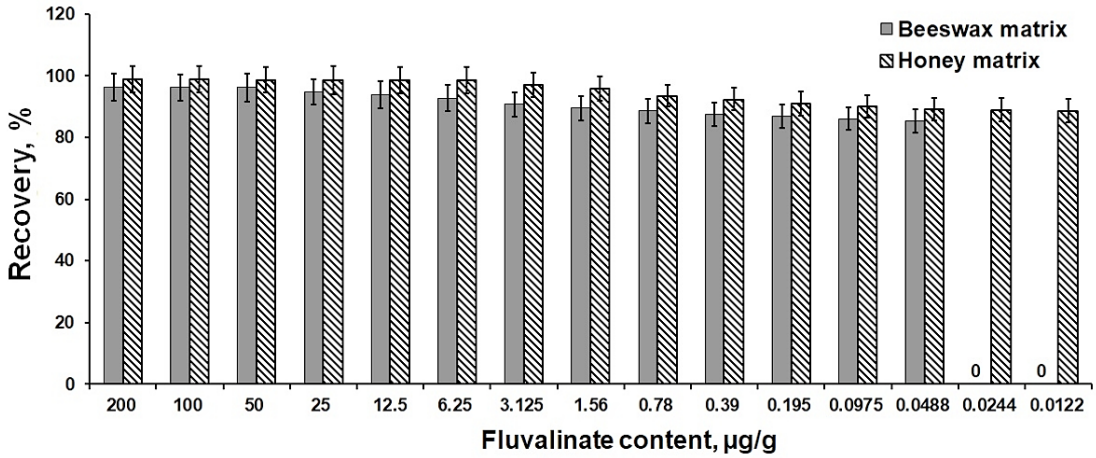

Fig. 7: Recovery of spiked fluvalinate concentrations

According to the Guideline on bioanalytical method validation and Russia State Pharmacopoeia XV, the recovery should be in the range of 80 to 120%. The results obtained allow us to conclude that this criterion is met.

Sensitivity

For the sensitivity study, the limit of detection (LOD) and limit of quantification (LOQ) of fluvalinate in both test matrices were determined (Table 6). The acceptance criteria for these parameters are as follows: the detection limit corresponds to a signal-to-noise ratio of 3:1, and the quantification limit corresponds to a value of 10:1.

Table 6: Limit detection and limit quantification results

Matrix	LOD, µg/g	LOQ, µg/g
Beeswax	0.02	0.05
Honey	0.005	0.012

As can be seen from the data provided, the values obtained are significantly lower than the MRLs established by EU, US and Australian legislation.[29]

Table 7: Repeatability and reproducibility results

Matrix	Repeatability (RSD, %)		Reproducibility (RSD, %)	
Beeswax	0.5 µg/g	0.05 µg/g	0.5 µg/g	0.05 µg/g
	2.1	2.7	4.0	4.5
Honey	0.12 µg/g	0.012 µg/g	0.12 µg/g	0.012 µg/g
	1.6	1.8	4.2	4.8

Repeatability and Reproducibility

In order to assess the reproducibility, ten samples of beeswax were subjected to spike with fluvalinate at two concentrations: 0.5 µg/g and 0.05 µg/g. Similarly, ten honey samples were spiked at concentrations of 0.12 µg/g and 0.012 µg/g. The repeatability was carried out in the same laboratory and on the same day, by the same operator, with the same equipment and reagents. The reproducibility test was performed in the same laboratory, on 3 different

days over 2 weeks, by the same operator, with the same equipment and reagents. The findings of these investigations are summarized in Table 7.

As can be seen, the repeatability and reproducibility of the method, expressed as relative standard deviation (RSD), were less than 5%.

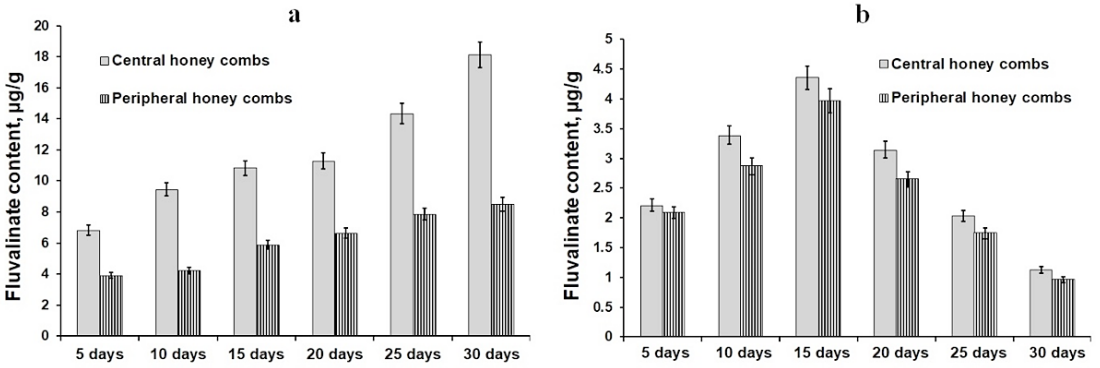

Fig. 8: Fluvalinate residues range in beeswax (a) and honey (b) samples, collected during treatment with acaricidal strips

Fluvalinate Accumulation in Beeswax and Honey Samples from Bee Hives

To study the accumulation and migration of fluvalinate in a beehive, beeswax and honey samples collected during veterinary treatment of bees with acaricidal strips containing fluvalinate as the active ingredient were analyzed (Fig. 8 a, b). Before the strips were placed in the hives, beeswax and honey samples were found free from fluvalinate.

It is evident that fluvalinate has been detected in all samples of beeswax collected, both from central and peripheral honey combs. There has been an increase in its concentration, indicating the accumulation of the acaricide in this matrix. It is worth noting that the concentration of fluvalinate in the central honey comb was higher than that in the peripheral one. Thus, on the thirtieth day, the concentration of the acaricide was 18.12 µg/g in the central honey comb, while in the peripheral honey comb it was only 8.49 µg/g.

During the first 15 days of the experiment, an increase in fluvalinate content was observed in honey samples, and then by the 30th day, a decrease to values of 1.12 µg/g and 0.96 µg/g in the central and peripheral combs, respectively.

Then, after removing the strips from the hives on the 30th day, the study of the samples was continued for up to 6 months, since there are no data in the literature sources on the study of fluvalinate accumulation over such a long period of time. Figure 9 (a, b) shows the results obtained.

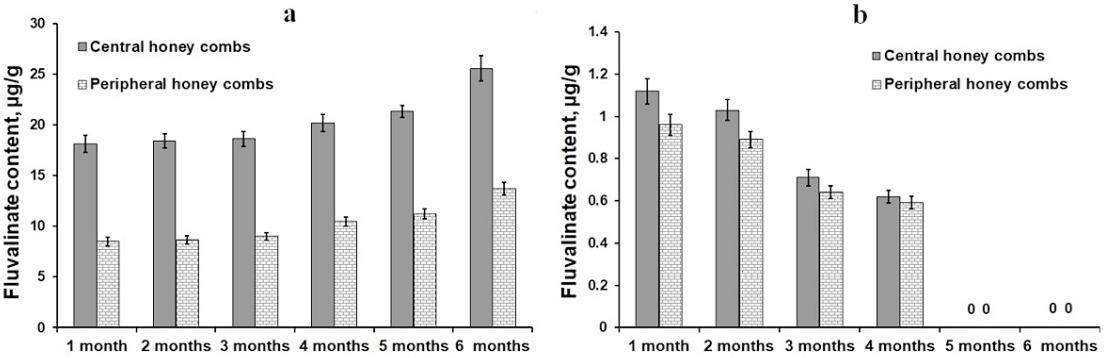

Fig. 9: Fluvalinate residues range in beeswax (a) and honey (b) samples, collected after treatment with acaricidal strips

As can be seen from the diagram, the experiment revealed a significant accumulation of fluvalinate in beeswax, reaching concentrations as high as 25.6 µg/g and 13.71 µg/g, respectively, in samples collected from the center and periphery of the honeycomb. Conversely, honey samples demonstrated a decline in acaricide levels, with fluvalinate undetectable in any sample by the fifth and sixth months. This phenomenon can be attributed to the migration of this lipophilic compound from honey into the beeswax.

Discussion

Fluvalinate is one of the most widely used acaricides against *Varroa destructor* and *Acarapis woodi* mites.[23,46] Since it is a non-volatile, lipophilic compound with a decomposition temperature of 200 °C,[47] its accumulation in bee products poses a serious danger.

Chromatographic techniques are employed for the detection of fluvalinate. We have developed a reversed-phase high-performance liquid chromatography with diode array detection (HPLC-DAD) method, which has a limit of quantification (LOQ) of 0.05 µg/g for fluvalinate in beeswax and 0.012 µg/g in honey. These results are an order of magnitude greater than those obtained by Adamczyk S. in the work,[23] where the LOQ for beeswax was 0.5 µg/kg and no analysis was performed for honey, and are comparable to the findings of Martel A.-C., who reported an LOQ of 0.01 µg/g for honey. The sensitivity of the HPLC approach proposed by Jamal M. was even higher, with an LOQ of 0.0053 µg/g, although it is worth noting that they did not analyze fluvalinate but another pyrethroid, flumethrin.[39] Our method involves extracting fluvalinate from the beeswax or honey matrices using LLE and subsequently employing HPLC-DAD for detection. The use of a dichloromethane:isooctane (2:8, v/v) solvent mixture in an appropriate volume as an extraction solution yielded positive results, with the addition of a three-step freezing process for beeswax samples prior to centrifugation, likely enhancing the extraction efficiency of the desired substance from the matrix. An additional purification step using cartridges, such as in the work of Adamczyk S.,[23] was not required, reducing potential losses. Filtration through a PTFE membrane was used to remove long-chain hydrocarbons.

The conducted studies demonstrated the accumulation of fluvalinate in beeswax after the treatment of bee colonies with acaricidal strips. Even after six months, relatively high levels of acaricide were detected in the samples. Lozano A. reported lower levels (29–454 µg/kg) of fluvalinate detected in beeswax and bee bread. The researchers noted a relationship between the lipophilicity of the compound and the presence of its residues in bee products after veterinary treatments.[48] Abd El-Wahab T.E. found fluvalinate residues (Mavrik 2F) in honey at a maximum concentration of 18.74 ppm already 24 h after bee treatment. After 90 days, the drug content decreased to 0.13 ppm. The same trend was observed for beeswax samples. However, the amount of fluvalinate detected in this matrix was higher than in honey.[32] There is an opinion that the presence of fluvalinate in beekeeping products is due to its accumulation inside the hive, in particular in beeswax honey combs used by beekeepers for a long time.[38,49,50] Thus, Mullin C.A. reported that the median concentration of fluvalinate in honeycombs is 3595 µg/kg.[51] In this aspect, the work of El Agrebi N. is of particular interest, in which the authors present data on the content of residues of veterinary drugs, including fluvalinate, in Belgian wax: brood comb wax, recycled comb wax, honey comb wax and cappings wax. Quite a high prevalence of fluvalinate residues was observed (from 65.5 to 100%), the maximum concentration of the acaricide was from 0.53 to 8.68 mg/kg, depending on the type of wax.[28] Researchers in Egypt found fluvalinate in 60% of beeswax samples at concentrations well above the MRL (50 µg/kg).[52] The authors suggested that this could be due to the uncontrolled use of acaricides when treating bees.[5] Also, a fairly high frequency of occurrence (50%) of fluvalinate was noted when monitoring Italian beeswax.[1]

Fluvalinate is a non-polar compound that is capable of dissolving in a hydrophobic medium, which is why its traces are rarely found in honey. As a result of monitoring a total of 101 honey samples from Galicia, only eleven of them were identified to contain fluvalinate at concentrations ranging from 10 to 40 mg/kg.[53] A study of honey samples from various regions of Egypt revealed the presence of 10 micrograms of fluvalinate per kilogram in one out of 64 samples examined.[37] In an analysis of 33 honey samples originating from different botanical

origins (acacia, chestnut and Manuka), Zheng W. and colleagues detected fluvalinate in only two samples. Additionally, they found that out of 10 royal jelly samples, none were contaminated with this acaricide.[36] Balayannis P.G., in his analysis of royal jelly for pesticide residues, also reported that none of the samples contained fluvalinate.[54]

Therefore, based on our data as well as the findings of other researchers, it can be concluded that fluvalinate accumulates in beeswax and can remain there for an extended period of time. This presents a significant threat to both bees and health of consumers. Contaminated beeswax can no longer be used for making wax foundation, as fluvalinate is a heat-resistant compound that can withstand temperatures up to 140 °C, which is the temperature at which beeswax is sterilized. Jamal M. suggests that the use of beeswax with flumethrin residue in cosmetology and pharmacology should be thoroughly investigated.[39] We agree that such research is also necessary when it comes to the presence of fluvalinate in beeswax. While the absence of fluvalinate buildup in honey is encouraging, its presence in this major beekeeping product following veterinary treatment of bees could also pose some risk.

The current difficult situation requires certain decisions to be made. Considering the widespread use of fluvalinate, as well as the high level of detectable residues, it seems appropriate to monitor beekeeping products, both in apiaries and during their commercial sale.

An alternative solution to this complex problem may be the use of acaricides of other classes, for example, organic acids (formic and oxalic). This approach is already being practiced in many countries around the world, including Russia.[55–58]

Conclusion

Thus, the developed HPLC-DAD method demonstrated a fairly high sensitivity for the detection of fluvalinate in beeswax and honey. The analysis of wax and honey collected from the apiary during and after veterinary treatment of bees against *Varroa* and *Acarapis* mites showed the presence of fluvalinate in all samples tested. However, in the 5th month of observation, the acaricide was no longer detected in honey samples, while an increase in its concentration was noted in beeswax samples, indicating the migration of fluvalinate from honey to beeswax and its accumulation in this matrix. The results obtained coincide with the data of other researchers. The solution to this problem can be monitoring bee products, as well as using environmentally friendly drugs based on organic acids to combat mite infestations.

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Conflict of Interest

The authors do not have any conflict of interest.

Data Availability Statement

The manuscript incorporates all datasets produced or examined throughout this research study.

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

Clinical Trial Registration

This research does not involve any clinical trials.

Permission to Reproduce Material from other Sources

The manuscript does not contain any materials that have been previously published elsewhere.

Author Contributions

- **Olga Gruznova:** Conceptualization, Methodology, Writing – Original Draft.
- **Dmitry Gruznov:** Data Collection, Analysis, Writing – Review and Editing.
- **Alexey Sokhlikov:** Investigation, Writing – Review and Editing.
- **Gulizar Shcherbakova:** Methodology,

Investigation, Validation.

- **Nikolay Popov:** Supervision, Project Administration, Validation.
- **Anton Lobanov:** Funding Acquisition, Resources, Project Administration.
- **Irina Chesnokova:** Methodology, Investigation, Validation.

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