Section A-Research paper

LC Method for Simultaneous Estimation of Fipronil and Methoprene in its combined Dosage Form.

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Running Title: HPLC estimation of Fipronil and Methoprene

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Abbreviations: ACN – Acetonitrile; ICH – International Council on Harmonization; LOD – limit of detection; LOQ – limit of quantitation; RSD – Relative standard deviation; RP-HPLC – Reverse phase-HPLC; SD – Standard deviation; UV – Ultraviolet, Fipro – Fipronil, Metho-Methoprene.

Keywords: Fipronil, Methoprene, HPLC, Method

ABSTRACT

The present study describes the systematic development and validation of RP-HPLC method for the estimation of Fipronil and Methoprene in its combined dosage form. The analysis was conducted using a Shimadzu Shim-pack GIST C18 column and Acetonitrile: Water (80:20, %v/v) as the mobile phase, with UV detection at 264nm. The method exhibited good separation, with retention times of 3.1 min for Fipronil and 4.71 min for Methoprene. The calibration curves showed excellent linearity over the concentration range of 20-100 µg/mL, with high correlation coefficients (R2) of 0.999 for Fipronil and 0.997 for Methoprene. Precision and robustness testing yielded %RSD values of less than 2%. The assay results for Fipronil and Methoprene were 99.02% and 99.63%, respectively, indicating high accuracy with significant % recovery. The method also met acceptance criteria for theoretical plates count, tailing factors, and other validation parameters as per ICH Q2 (R1) guidelines. Therefore, this accurate and precise RP-HPLC method can be reliably used for routine analysis of Fipronil and Methoprene in quality control laboratories.

1. INTRODUCTION

Fipronil and Methoprene, sold under the brand name Frontline Plus®, are utilized for managing and treating flea and tick infestations in dogs and cats at all stages. Fipronil belongs to the phenylpyrazole chemical family and is a highly effective pesticide. Its mode of action involves inhibiting chlorine ions from binding to the GABA neurotransmitter in both presynaptic and postsynaptic regions. It appears as a white powder with a moldy smell and was initially approved for use in the United States in 1996. Chemically, it is referred to as (5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole)[1,2].

Methoprene is an insecticide effective against insects. Its chemical name is isopropyl(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienoate. It is commonly used for surface substrate

treatment and effectively controls R. dominica (a type of stored grain pest) and external feeders of stored grains. Additionally, exposure to Methoprene can reduce the fertility of adult insects that come into contact with it. Adding a neurotoxic contact pesticide could hinder egg-laying in other parts of the food facility by immobilizing insects exposed to the treated grain, preventing them from moving away. There are two variants of the pesticide, namely s-methoprene and r-methoprene, with s-methoprene behaving like a crucial hormone in insects. It proves effective against various insects, including fleas, flies, moths, beetles, and others. Methoprene was initially approved for use in the United States in 1975, while s-methoprene was registered later in 1985 [3,4].

Fipronil elicits diverse responses in invertebrates. It acts by binding non-competitively to GABA (gamma-aminobutyric acid) receptors and two types of glutamate-gated chloride channels (GluCl-D and GluCl-N) present in the invertebrate central nervous system. This binding inhibits the entry of chloride ions into nerve cells, preventing overexcitation and its subsequent effects (Tingle et al., 2003). Fipronil's effectiveness in preventing drug resistance is attributed to its three distinct sites of action. Furthermore, since mammals do not possess GluCl receptors and there are structural differences in GABA receptors between invertebrates and mammals, Fipronil exhibits exceptional selectivity towards invertebrates. As a result, the likelihood of developing drug resistance is considered low [5-9].

Methoprene functions as an insect growth regulator, disrupting hormonally and enzymatically controlled processes that are specific to insect physiology. Among the widely used and successful insect growth regulators, Methoprene acts as a juvenile hormone agonist, preventing the emergence of offspring in target insects without causing harm to the adult insects. Formulated pesticides that target enzymatic or hormone-regulated processes in insects usually show minimal toxicity to non-target organisms. Nevertheless, Methoprene has been associated with negative effects, including developmental abnormalities. At higher concentrations, certain fish species and non-target aquatic organisms, such as insects, crabs,

and other creatures, have been observed to suffer harm due to the presence of this insecticide [10-14].

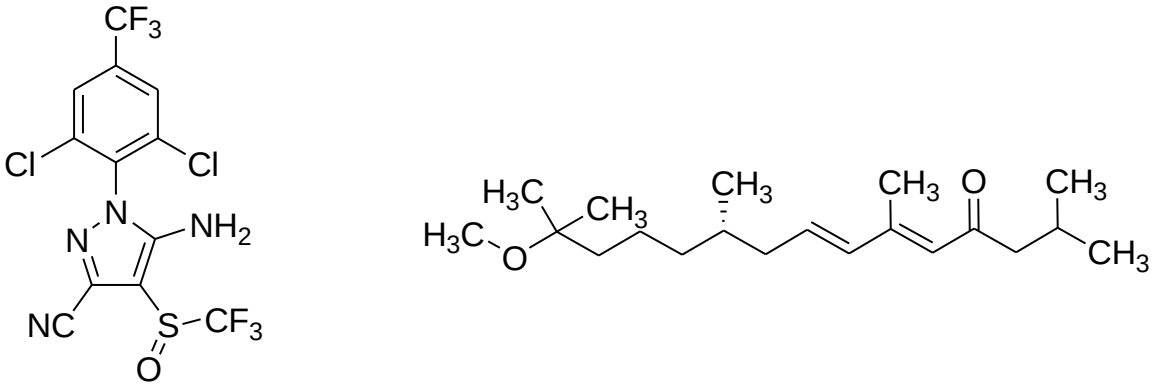

Figure 1. Chemical structure of Fipronil and Methoprene.

The scientific community is highly interested in measuring Fipronil and Methoprene due to their potential health advantages. Over time, several analytical methods have been created to determine these compounds separately or together from various sources. However, as of now, there are no reported analytical methods available for simultaneously quantifying both Fipronil and Methoprene.

This study's primary objective is to develop and validate an RP-HPLC method for measuring Fipronil and Methoprene when combined in a dosage form. The proposed method seeks to ensure precise and dependable quantification of these bioactive substances. By thoroughly examining and assessing different parameters related to HPLC method development and validation, the research aims to establish an optimized protocol suitable for routine analysis of these compounds.

2 MATERIAL AND METHOD

2.1 Instrumentation

The RP-HPLC analysis was performed using a Shimadzu instrument equipped with a UV detector. Data processing was conducted using Lab solutions Software. The study employed a

Shimadzu C18 Shim-pack Gist column (250 × 4.6 mm, 5µm) as the stationary phase. For mobile phase filtration, a vacuum pump filtration assembly (Rocker 300) was used, and a Sonicator water bath from Janki Impex was utilized. Measurements were taken with a UV–spectrophotometer 1800 from Shimadzu Japan. Additionally, an analytical weighing balance from Mettler Toledo and a pH meter were employed.

2.2 Chemicals and reagents

Fipronil and methoprene were provided as gift samples by Sava Healthcare Ltd. in Surendranagar, Gujarat, India. HPLC-grade methanol, acetonitrile (ACN), and water were purchased from Fine Chem in Mumbai, India. Analytical-grade glacial acetic acid and ortho-phosphoric acid were obtained from Rankem Laboratories Private Ltd in Thane, Maharashtra, India. For filtration, a 0.45 µm pore size membrane filter and a 0.22 µm test filter paper were acquired from Pall India Private Ltd in Andheri, Mumbai, India.

2.3 Analytical method development and chromatographic condition

The method development process comprised various optimization stages to achieve specific separation of the targeted drugs. These optimization steps involved selecting the right solvent, a suitable column phase, an appropriate mobile phase, and an optimal detecting wavelength. Different parameters were carefully examined to identify the best conditions for each step, and numerous trials were conducted to find the most efficient combination of these parameters. Following extensive optimization, the developed chromatography method exhibited outstanding theoretical plate count, peak resolution, and symmetry, allowing for the simultaneous quantification of all three selected drugs. The optimized method ensures dependable and precise results, making it well-suited for routine analysis and quality control of products containing these compounds. The optimized chromatographic conditions are summarized in Table 1.

2.3.1 Preparation of standard stock solution

To prepare standard stock solutions of Fipronil and Methoprene, 10 mg of each drug was taken and dissolved in a 10 mL volumetric flask to obtain a concentration of 1000 µg/ml for each solution. From these stock solutions, a series of working standard solutions were prepared by diluting the appropriate volumes, resulting in concentrations ranging from 10 to 100 µg/ml.

2.3.2 Chromatographic Conditions:

For chromatographic separation of Fipronil and Methoprene, a Shimadzu Shim-pack GIST C18 column (250 × 4.6 mm, 5 µm particle size) was employed. Acetonitrile was chosen as the diluent and delivered at a flow rate of 1.5 ml/min. The mobile phase used for injection consisted of a mixture of acetonitrile and water in a ratio of 80:20 v/v.

2.4 Validation of HPLC method

Validation of the developed HPLC method was carried out according to the ICH Q2 (R2) guideline [15].

2.4.1 Specificity

Specificity refers to the analytical method's capacity to accurately and specifically measure the target analyte in the presence of other components, such as impurities, degradation products, and matrix elements that may exist in the sample matrix. To assess the method's specificity, the chromatograms of both the standard and marketed formulation were compared to identify any interference from excipient peaks. Additionally, a blank chromatogram was recorded to check for any unwanted interferences [16].

2.4.2 System suitability

The suitability of the method was verified by calculating several chromatographic parameters, including retention time, number of theoretical plates (N), resolution (Rs), and tailing factor (Tf) from the standard solution chromatogram. The results of these system suitability parameters indicated that the chosen chromatographic conditions were appropriate for the method [17].

2.4.3 Linearity

To establish a calibration range, appropriate aliquots from the working standard solutions of the studied drugs were utilized. These solutions were then injected into the HPLC system and analysed according to the specified chromatography conditions. The linearity range for Fipronil was found to be 20-100 µg/ml, while for Methoprene, it was 20-100 µg/ml. A calibration curve was plotted using peak area versus the corresponding concentration of each drug, and the regression line equation and correlation coefficient were calculated to assess the linearity (Table 2) [18,19].

2.4.4 Precision:

Precision in an analytical method refers to the degree of agreement among multiple measurements obtained from several samplings of the same homogeneous sample under specific conditions. Precision was assessed in terms of repeatability, intra-day precision, and inter-day precision. Intra-day precision was evaluated by analyzing sample solutions containing a mixture of the compounds (at concentrations of 20, 30, and 40 µg/ml) three times on the same day (n = 3), covering the entire range of the calibration curve. To assess inter-day precision, sample solutions containing a mixture of the compounds (at concentrations of 20, 60, and 80 µg/ml) were analyzed at three different concentration levels, spanning the entire range of concentrations, over a period of three consecutive days (n = 3). From the obtained peak areas, the mean, standard deviation (SD), and relative standard deviation (% RSD) values were calculated [20].

Repeatability of measurement of peak area ware determined by analyzing middle concentration of 60 µg/mL for six times.

2.4.5 Accuracy:

Accuracy in an analytical method refers to how close the measured values are to a conventional true value or a conventional reference value. To estimate the accuracy of the method, % recovery was calculated. This was achieved through the standard addition method, where known amounts of standard spikes were added at three different levels (50%, 100%, and 150%) to a pre-quantified sample solution [21,22].

2.4 .6 ROUSTNESS:

The ability of an analytical method to remain unaffected by minor intentional variations in method parameters, indicating its consistency, is known as robustness. In this study, robustness was evaluated by making small deliberate changes in the wavelength of detection (±1nm) and flow rate (±0.1mL/min). The %RSD for the peak area was calculated to assess the method's robustness [23,24].

2.4.7 LOD&LOQ:

To assess the sensitivity of the developed method, the LOD (Limit of Detection) and LOQ (Limit of Quantification) were determined. The linearity of the standards was tested three times to obtain the standard deviation of the intercept (SD) and the slope of the regression equation (S) value. The calibration curves were repeated five times, and the standard deviation of the intercept was calculated. Using this information, the LOD and LOQ were then calculated using the appropriate equations [25,26].

$$LOD=3.3\times(SD/Slope)$$

LOQ=10×(SD/Slope) Where, the SD = Standard deviation of the Y-intercepts of the five calibration curves. Slope=Means lope of the five calibration curves.

3 RESULTS AND DISCUSSION

3.1 Optimization of chromatographic conditions

Various solvent proportions, including buffers of different pH and concentration, methanol, acetonitrile (ACN), and water, were experimented with in different ratios, but satisfactory results were not achieved. However, a mobile phase composition containing ACN: Water (80:20 %, v/v) provided optimal separation. With this mobile phase, retention times of 3.1 min and 4.71 min were observed for Fipronil and Methoprene, respectively, with good resolution. The peak shape was appropriate, and the tailing factor and theoretical plate were satisfactory. Thus, this mobile phase was chosen as the optimized one.

Table 1: Optimized Chromatographic Condition

Column	Shimadzu Shim-pack GIST C18 column, (250*4.6, 5µ)
Flow rate	1.5 ml/min
Injection volume	10 µL
Mobile phase	Acetonitrile: Water (80:20) v/v
Detector	UV
Detection Wavelength	264 nm

3.2 Selection of detection wavelength

Choosing the appropriate detection wavelength is crucial for enhancing the sensitivity of the method. In this study, standard solutions of Fipronil and Methoprene were scanned in the UV region, ranging from 200 nm to 400 nm. Upon analysing the overlay spectra, it was observed that both compounds exhibited absorbance at 264 nm. As a result, 264 nm was selected as the optimal wavelength for the detection of the drugs, as shown in Figure 2A.

3.3 Validation of the developed analytical method

3.3.1 Specificity

To assess specificity, chromatograms of the placebo and synthetic mixture were compared, as depicted in Figure 2. Upon examination, it was evident that all the prodrug components were distinctly detected without any interference from the sample matrix or blank.

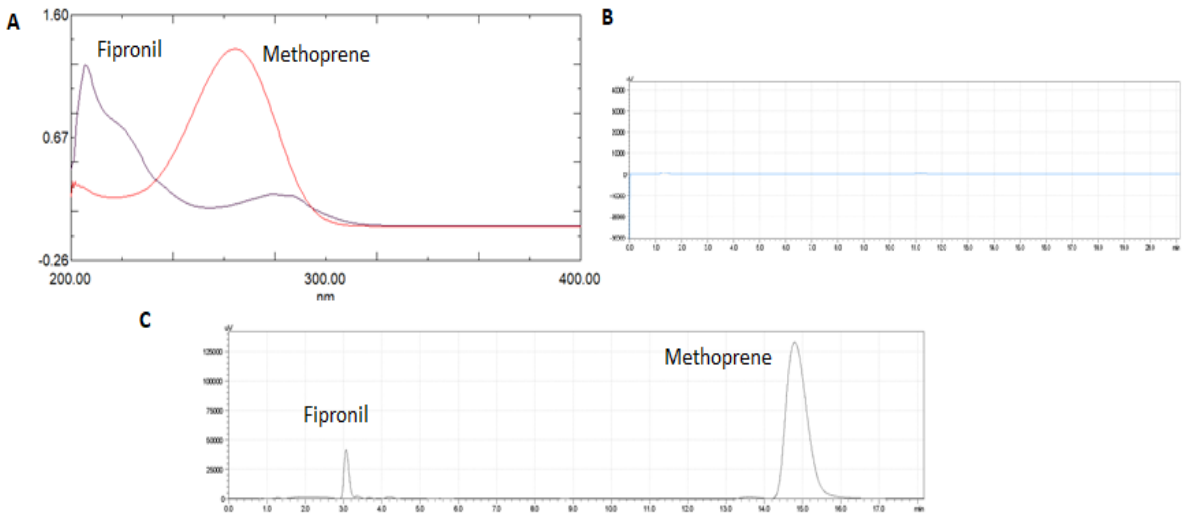

Figure 2. (a) Overlay UV spectra of Fipronil and Methoprene. (b) Chromatogram of Placebo. (c)Combined chromatogram of mixture of Fipronil and Methoprene

3.3.2 System suitability

The system suitability of the method was verified by calculating different chromatographic parameters, including retention time, number of theoretical plates (N), resolution (Rs), and

tailing factor (Tf) from the chromatograms of standard solutions. The results of these system suitability parameters confirmed that the selected chromatographic conditions were suitable and appropriate for the method.

3.3.3 Linearity and range

The calibration curve was constructed by plotting the concentration of drugs against their respective peak areas. Standard solutions of drugs ranging from 20 to 100 µg/ml were prepared, and 10 µL of each solution was injected into the HPLC column. Linearity was assessed using linear regression analysis, and the method showed linear behaviour in the concentration range of 20–100 µg/mL (n = 5) for both Fipronil and Methoprene. The correlation coefficients (R2) obtained for Fipronil and Methoprene were 0.999 and 0.997, respectively.

3.3.4 Limit of detection and limit of quantification

The LOD and LOQ of the method were determined by utilizing the standard deviation (SD) of the Y-intercept of the calibration curve and the mean slope of the calibration curve. The lowest detectable amounts of fipronil and methoprene were found to be 0.57 µg/ml and 0.21 µg/ml, respectively, while the limit of quantification (LOQ) for fipronil and methoprene were determined to be 1.69 µg/ml and 0.65 µg/ml, respectively

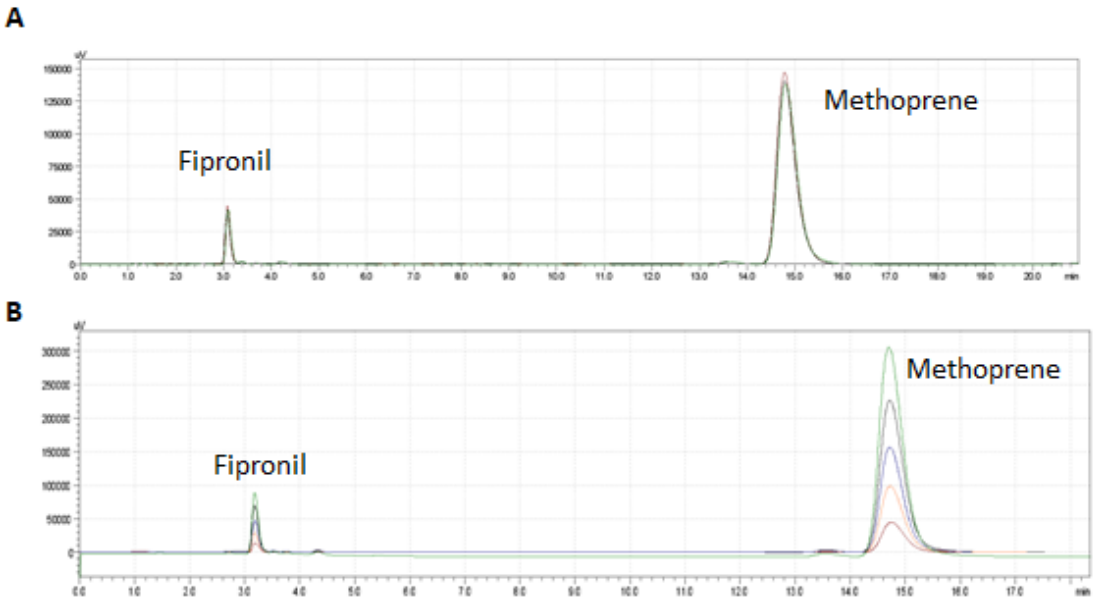

Figure 3. (A) Chromatogram of System suitability. (C) Chromatogram of Linearity.

Table 3: Data of Linearity, LOD and LOQ

Name of Drug	Conc. Range(µg/ml)	Equation	Regression coefficient	LOD	LOQ
Fipronil	20-100	y = 4818x+5887	R^2 = 0.999	0.57 µg/ml	1.69 µg/ml
Methoprene	20-100	y = 8325x+16580	R^2 = 0.997	0.21µg/ml	0.65 µg/ml

3.3.5 Accuracy

Accuracy of an analytical method refers to how close the practical value is to the true value (100%). To evaluate accuracy, a known amount of standard was added (spiked) to pre-analyzed samples at three different concentration levels (50%, 100%, and 150%). The percentage (%) recovery was determined to be in the range of 98.16-98.45% for fipronil and 98.36-100.37% for Methoprene (Table 4). These results indicate that the method is accurate and provides reliable measurements.

Table: 4 Data of Accuracy Study

Drug	Level	Amount of sample µg/ml	Amount of standard spike µg/ml	Total amount (µg/ml)	Amount found (µg/ml)	% Recovery S.D.
FIPRO	50% (n=3)	9	4	13	11.45	98.16±0.035
	100% (n=3)	9	8	17	15.69	98.06±0.045
	150% (n=3)	9	12	21	19.69	98.45±0.040
METHO	50% (n=3)	8	2	10	5.95	99.16±0.017
	100% (n=3)	8	4	12	8.03	100.37±0.01
	150% (n=3)	8	6	14	9.63	98.36±0.032

3.3.6 | Precision

To evaluate the repeatability of the method, a 60 µg/mL solution was injected multiple times. The average, standard deviation (SD), and %RSD of the area were calculated and reported. Intra-day precision was assessed by injecting the middle three concentrations of the analytical procedure within a day, using the same experimental conditions. On the other hand, inter-day precision involved analyzing the method on different days in the same laboratory. The

percentage (%RSD) values of the response for both intra-day and inter-day precision were found to be less than 2%, indicating that the method is highly precise and consistent (Table 4).

3.3.7 | Robustness

The drugs were tested at three different wavelengths and three different flow rates. The %RSD was calculated and found to be less than 2%, indicating the robustness of the proposed method. A summary of the validation parameters can be found in Table 4.

Table 4: Data of Precision and Robustness.

		Fipro (%RSD)	Metho (%RSD)
Precision	Repeatability (n=6)	0.5535	1.33271
	Interday (n=3)	1.1351	0.5536
	Intraday (n=3)	0.4365	0.3672
Robustness	Change in wavelength	1.568	0.7859
	Change in Flowrate	1.4896	0.8569

3.3.8 Analysis of marketed formulation:

The % assay of the Fipronil and Methoprene synthetic mixture was determined to be 99.02% and 99.63%, respectively. Moreover, the % amount of Fipronil and Methoprene in the marketed formulation was found to be within the acceptable limits. The analysis revealed no interference from any of the excipients typically present in the proposed formulation.

4 CONCLUSION

Method was successfully developed for Simultaneous determination of Fipronil and Methoprene. This method was successfully applied for convention analysis of Fipronil and Methoprene in combined marketed dosage form with results in compliance with the standards. The systematic approach was utilized for Method development and to validate All the Validation parameter were found within the acceptance criteria according to ICH Q2(R2) guideline. The proposed Method was simple, rapid, accurate, precise and specific and have the ability to determine Fipronil and Methoprene in the dosage form. s. The developed method can be a helpful tool for the analysis of sample during quality control of formulations for routine analysis in the pharmaceutical industry.

REFERNCES:

1. Gonçalves S, Vasconcelos MW, Mota TF , Identifying global trends and gaps in research on pesticide fipronil: a scientometric review. *Environ Sci Pollut Res.* 2022;29: 79111–79125.

2. Gupta RC, Anadón A, Fipronil. In Veterinary toxicology 2018 Jan 1 (pp. 533-538). Academic press.

3. Arthur FH, Athanassiou CG, Morrison WR, Mobility of Stored Product Beetles after Exposure to a Combination Insecticide Containing Deltamethrin, Methoprene, and a Piperonyl Butoxide Synergist Depends on Species, Concentration, and Exposure Time. *Insects.* 2020;11(3):151. https://doi.org/10.3390/insects11030151

4. Rehbein S, Capri B, Duscher G, Keidane D, Kirkova Z, Petkevicius S, Rapti D, Wagner A, Wagner T, Chester ST, Rosentel J, Efficacy against nematode and cestode infections and safety of a novel topical fipronil, (S)-Methoprene, eprinomectin and praziquantel

combination product in domestic cats under field conditions in Europe. *Veterinary Parasitology*. 2014;28:10-7.

5. Monteiro JP, Oliveira PJ, Moreno AJ, Jurado AS, Disruption of hepatic mitochondrial bioenergetics is not a primary mechanism for the toxicity of methoprene–relevance for toxicological assessment. *Chemosphere*. 2008;72(9):1347-54.

6. U.S. Food and Drug Administration. Guidance for Industry: Analytical Procedures and Methoprene Validation for Drugs and Biologics. Available at: https://www.fda.gov/media/121365/download.

7. Oudejans L, Mysz A, Snyder EG, Wyrzykowska-Ceradini B, Nardin J, Tabor D, Starr J, Stout II D, Lemieux P. Remediating indoor pesticide contamination from improper pest control treatments: Persistence and decontamination studies. Journal of hazardous materials. 2020,5;397:122743.

8. Wick K, Bond C, Buhl K, Stone D. Methoprene General Fact Sheet; National Pesticide Information Center, Oregon State University Extension Services.

9. Hafeez A, Tawab IA, Development and Validation of an HPLC Method for the Simultaneous Determination of Fipronil, Chlorfenapyr, and Pyriproxyfen in Insecticide Formulations. *J. of AOAC Int.* 2016;99(5):1185-1190.

10. Iqbal S. Development and Validation of HPLC Method for the Simultaneous Determination of Insecticides (Carbofuran and Fipronil) in its Technical and Granular Formulation. Pakistan Journal of Analytical & Environmental Chemistry. 2020 Dec 21;21(2):247-53.

11. Saito I, ueno E, oshima H, matsumoto H, sasaki K, maitani T. [Analytical method for methoprene in foods using HPLC]. Shokuhin eiseigaku zasshi. 2006 aug;47(4):173-7. Japanese. Doi: 10.3358/shokueishi.47.173. Pmid: 16984038.

12. Yang SS. A rapid method for the determination of methoprene in tobacco by high performance liquid chromatography. Chromatographia. 1992 Apr;33:309-12.
13. Iqbal S. Development and Validation of HPLC Method for the Simultaneous Determination of Insecticides (Carbofuran and Fipronil) in its Technical and Granular Formulation. Pakistan Journal of Analytical & Environmental Chemistry. 2020 Dec 21;21(2):247-53.
14. Oltean EG, Nica A. Development and validation of a RP–HPLC method for the quantitation studies of fipronil in Parakill suspensions. Medicamentul Veterinar. 2011.
15. Guideline, ICH Harmonised Tripartite. "Validation of analytical procedures: text and methodology." *Q2 (R1)* 1.20 (2005): 05.
16. Saito I, ueno E, oshima H, matsumoto H, sasaki K, maitani T. [Analytical method for methoprene in foods using HPLC]. Shokuhin eiseigaku zasshi. 2006 aug;47(4):173-7. Japanese. Doi: 10.3358/shokueishi.47.173. Pmid: 16984038.
17. Yang, S.S. A rapid method for the determination of methoprene in tobacco by high performance liquid chromatography. *Chromatographia* **33**, 309–312 (1992). Https://doi.Org/10.1007/bf02275907
18. Riviere JE, Papich MG. Pharmacology and Therapeutics, 9th edition, Wiley-Blackwell, The United States of America, 2009, pp. 1187.
19. Maddison JE, Stephen W, Small Animal Clinical Pharmacology, Elsevier Limited, 2008, pp. 226.
20. Ramasubramanian T, Paramasivam M, Jayanthi R, Chandrasekaran S. A simple and sensitive single-step method for gas chromatography-mass spectrometric determination of fipronil and its metabolites in sugarcane juice, jaggery and sugar. Food Chem. 2014; 150:408-413.

21. Farouk M, Hussein LA, El Azab NF. New HPLC and fluorimetric methods for the determination of pyriproxyfen and pyridalyl insecticide residues in tomatoes. J AOAC Int. 2014; 97(1):188-96.
22. Bhavsar P, Jha LL, Patel L, Tandel F. LC method for the quantification of Quercetin, Berberine, and Phytosterol in lyophilized liposome. Sep Sci plus. 2023; e2300047. https://doi.org/10.1002/sscp.202300047.
23. Payal JP, Vandana B Patel, Dimal A Shah, Stability indicating LC method for estimation of valbenazine and its degradation kinetic study. *Sep. Sci. plus.* 2020:*3(4)*:1-8.
24. Mahmoudi A, Efficient and simple HPLC method for spiramycin determination in urine samples and in pharmaceutical tablets. *Sep. Sci. plus.* 2018;*2(3)*:253-260.
25. Patel L, Shah D, Bhavsar P, Patel Z, Shah Z. Thin layer chromatography- densitometric method for quantification of apigenin, oleanolic acid, and β- sitosterol in clerodendrum serratum (Bharangi) root extract. Separation Science Plus.:2300038.
26. Khristi A, Jha LL, Dharamsi A. RP-HPLC Method Development and Validation for Simultaneous Estimation of Thymol, Eugenol and Alliin in Bulk and Novel Nanoformulation. Research Journal of Pharmacy and Technology. 2022;15(12):5761-6.