

Rapid isocratic HPLC determination of fipronil, (S)-methoprene, and major related substances using an end-capped monolithic column

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Abstract

In this study, a simple and rapid isocratic reversed-phase high-performance liquid chromatography (HPLC) method was developed. This method allows the simultaneous determination of active substances and their major related substances, fipronil sulfone and cis-methoprene, in spot-on products containing fipronil and (S)-methoprene. Separation was achieved using an end-capped monolithic column, with an isocratic mobile phase composed of acetonitrile and 0.1% formic acid (70:30, v/v) at 1.0 mL/min and UV detection at 245 nm. Compared to conventional particulate columns, this method provides significant advantages, including very low system back-pressure (~13 bar) and a short analysis time (~12 min). The method was validated for selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ), and system suitability. Good linearity was obtained ($R^2 \geq 0.995$), and repeatability (%RSD < 2.0) and recovery values ranged from 95.68% to 99.07%. LOD for fipronil, fipronil sulfone, cis-methopren, and trans methopren respectively 0.005 µg/mL, 0.007 µg/mL, 0.013 µg/mL, and 0.008 µg/mL. LOQ for fipronil, fipronil sulfone, cis-methopren, and trans methopren respectively 0.015 µg/mL, 0.021 µg/mL, 0.040 µg/mL, and 0.024 µg/mL. The developed method LOD and LOQ enabled reliable determination of the major related substances at concentrations below 0.05%, demonstrating its suitability for routine quality control. The environmental performance of the analytical procedure was evaluated using the AGREE and ComplexGAPI metrics. The proposed method achieved an AGREE score of 0.78 and an estimated E-factor of approximately 10.5, confirming good compliance with Green Analytical Chemistry principles through reduced solvent consumption, short analysis time, and low energy demand. The validated method was successfully applied to ten commercial veterinary spot-on products marketed in Türkiye, all of which complied with the specified acceptance criteria for active substance content and major related substances. The method provides a practical, reliable, and sustainable tool for routine quality control of veterinary medicinal products.

Keywords: Fipronil, green analytical chemistry, HPLC-DAD, (S)-methoprene, monolithic column, related substances

1. Introduction

Fipronil is a broad-spectrum phenylpyrazole insecticide widely used for the control of ectoparasites in companion animals [1]. (S)-methoprene is a broad-spectrum insect growth regulator [2]. Spot-on formulations used in cats and dogs provide comprehensive ectoparasite control by combining the adulticidal activity of fipronil with the ability of (S)-methoprene to inhibit the development of immature flea stages [3].

In veterinary medicinal products, exposure to various environmental conditions during the period from manufacturing to end use may lead to a decrease in the active substance content, resulting in the formation

of related substances and degradation products [4,5]. In post-marketing surveillance, the pharmaceutical quality of products is evaluated by regulatory authorities, and their efficacy, quality, and safety are rigorously assessed [6,7]. Control of both active substance content and impurities (including related substances and degradation products) within specified limits is essential to ensure product reliability [4,5]. Therefore, monitoring impurities in finished products under current pharmacopoeial standards and regulatory guidelines is a legal requirement [5,7,8].

Fipronil sulfone is one of the major related substances formed during the manufacturing and storage of fipronil

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and exhibits approximately tenfold higher neuroactivity in mammals compared to the parent compound [9]. (S)-methoprene exists as trans and cis geometric isomers. Under sunlight exposure, the trans isomer can undergo photoisomerization to form the biologically largely inactive cis isomer [2,10–12]. To prevent loss of efficacy, the cis isomer content is expected to remain below 5% according to stability specifications [3]. In addition, the requirement for active substance content to remain within $\pm 10\%$ of the labeled amount throughout the shelf life, combined with the risk of product recalls due to out-of-specification results, necessitates strict quality control procedures [7,13,14].

Various liquid chromatography-based methods have been reported in the literature for the determination of fipronil and/or (S)-methoprene. The European Pharmacopoeia (EP) recommends an isocratic method using a 150 mm end-capped ODS column with a relatively complex mobile phase for the determination of fipronil-related substances [8]. Although alternative UPLC methods offer shorter analysis times (approximately 7 min), they require high-pressure instrumentation and specialized column technologies [15]. Other reported HPLC methods either involve long analysis times (45–100 min) and complex gradient elution or provide limited information on impurity profiling [16–19]. Notably, there is currently no specific pharmacopoeial monograph addressing the simultaneous determination and major related substance profiling of the fipronil/(S)-methoprene combination [8]. Limited studies have reported simultaneous determination of fipronil and (S)-methoprene, while impurity profiling using a monolithic column and green analytical chemistry evaluation remain scarcely investigated [17,18].

Monolithic HPLC columns, because of their highly porous structure and large flow through channels, enable operation at significantly lower back-pressure even at high flow rates compared to conventional particulate columns. In addition, end-capping technology reduces silanol interactions, thereby improving peak symmetry [20]. In recent years, increasing environmental awareness has driven the integration of GAC principles into analytical workflows, promoting the development of sustainable methods with reduced solvent consumption and lower energy requirements [21,22]. The low backpressure provided by monolithic columns not only enhances analytical efficiency but also contributes to reducing the environmental footprint of analytical processes, demonstrating strong alignment with GAC principles [20].

Among the available GAC assessment tools, AGREE and ComplexGAPI provide comprehensive and

standardized metrics for evaluating the environmental performance of analytical methods and were therefore selected in the present study [21,22].

In this context, currently available analytical methods are associated with certain limitations, such as long analysis times, high system back-pressure, or insufficient capability for impurity profiling. Although simultaneous HPLC methods have been reported, the combination of an end-capped monolithic column with rapid isocratic separation, determination of the major related substances, and systematic evaluation using AGREE and ComplexGAPI metrics has not been previously described.

Therefore, the aim of this study was to (i) develop a rapid isocratic RP-HPLC method using an end-capped monolithic column for the simultaneous determination of fipronil, (S)-methoprene, and their major related substances, (ii) validate the method according to the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) guidelines, and (iii) evaluate its environmental performance using AGREE and ComplexGAPI metrics.

2. Materials and methods

2.1. Reference standard and chemicals

Certificated reference standard fipronil (99.8%, EDQM, Strasbourg, France), fipronil sulfone (99.7%, Dr. Ehrenstorfer, Augsburg, Germany), and (S)-methoprene (99.5%, Sigma-Aldrich, Saint Louis, MO, USA) were obtained from approved suppliers. HPLC-grade acetonitrile (Sigma-Aldrich) and formic acid (Merck, Darmstadt, Germany) were also obtained from approved suppliers. Water was produced using an ultra-pure water system (Flex-3, ELGA, UK). For the placebo: technical-grade butylhydroxyanisole (Merck, Darmstadt, Germany), butylhydroxytoluene (Merck, Darmstadt, Germany), polysorbate 80 (Croda, Chocques, France), Povidone K-25 (BASF, Ludwigshafen, Germany), ethanol (Commercial Alcohols, Ontario, Canada), and diethylene glycol monomethyl ether (Gattefosse, Saint-Priest, France) were procured and used.

2.2. Sample collection method

The study evaluated 10 spot-on veterinary products containing a fixed-dose combination of fipronil and (S)-methoprene. These samples were collected between 2024 and 2026 by the Republic of Türkiye Ministry of Agriculture and Forestry during routine pharmaceutical quality control inspections. A random sampling approach was employed to ensure that the selection represented various manufacturers within the

authorized legal supply chain. The examinations and analyses were carried out in an official laboratory

methoprene, spiking was performed based on the natural cis ratio stated in the reference standard

Table 1. Separation and pressure performance summary of the tested column for method development.

Column type	Dimensions (mm)	Stationary phase / particle size	Back-pressure (bar)	Analysis time* (min)	Critical chromatographic observation
Phenomenex Prodigy ODS3	250 × 4.6	5 µm particulate	80-100	18	Fipronil sulfone was not resolved from fipronil
ACE 5 C18	250 × 4.6	5 µm particulate	70-100	32	Related substances were not adequately separated
Phenomenex SphereClone Phenyl	250 × 4.6	3 µm particulate	60-80	4	Active ingredients and related substances co-eluted
Merck Purospher Star RP-18e	250 × 4.6	5 µm particulate	80-100	>40	Inadequate retention of (S)-methoprene
Supelco Discovery C18	100 × 4.6	5 µm particulate	30-40	14	Related substances were not completely resolved
Merck Chromolith RP-18e	100 × 4.6	Monolithic	12–15	12	**Resolution= 3.7 (fipronil/fipronil sulfone); 2.3 (cis/trans-methoprene)
Waters Acquity UPLC C18	100 × 2.1	1.7 µm particulate	-	-	Operating pressure exceeded acceptable limit at a flow rate of 1.0 mL/min

* Analysis time corresponds to the retention time of the last detected chromatographic peak.

**Resolution values are reported only for the selected Chromolith RP-18e column because complete chromatographic separation of the critical analyte pairs could not be achieved with the remaining stationary phases.

serving as a national reference, which operates under a quality management system compliant with the ISO/IEC 17025 standard. The findings regarding the products, whose names were anonymised, were evaluated for compliance with the guidelines [4], pharmacopoeia [8], and product specification limits (Table 1).

2.3. Preparation of standard solutions, placebo, spiked samples, and unlicensed product samples

Stock standard solutions were prepared separately from fipronil, fipronil sulfone, and (S)-methoprene reference standards at a concentration of 1000 µg/mL in acetonitrile.

A mixed working standard solution containing concentrations of 100 µg/mL fipronil, 2–5 µg/mL fipronil sulfone, and 120 µg/mL (S)-methoprene (predominantly the trans isomer) was prepared by diluting these stock solutions.

Taking into account the cis-methoprene content specified in the certificate of the (S)-methoprene reference standard, it was used for the identification of the cis-methoprene peak, selectivity studies, peak purity index assessment (≥ 0.99), and in the preparation of low-level spiked samples.

For fipronil sulfone, separate working solutions in the range of 0.5–10 µg/mL were additionally prepared to carry out calibration curves and recovery studies.

The placebo solution was prepared using excipients reported in commercial product formulations at the ratios specified in the product summaries [3].

Spiked samples were prepared at 80%, 90%, 100%, 110%, and 120% levels of the active ingredients by adding appropriate volumes of the working standard solution to the placebo matrix (for fipronil sulfone, at low levels within the 0.5–2.0% range). For cis-

certificate.

Commercial product samples (100 mg/mL fipronil and 120 mg/mL (S)-methoprene) were diluted with acetonitrile at a ratio of 1:1000 (v/v). Stock and working standard solutions, placebo samples, spiked samples, and commercial product samples were vortex-mixed for 30 s and sonicated for 5 min to ensure complete homogenization before filtration. All solutions were subsequently filtered through a 0.45 µm PTFE membrane filter and transferred to amber-coloured glass vials prior to HPLC analysis. Each sample was analysed in triplicate.

2.4. Instrumentation and chromatographic conditions

All chromatographic analyses were performed using an HPLC system equipped with a diode array detector (DAD) (Thermo Scientific Dionex Ultimate 3000) and Chromeleon™ 7.2.9 software (Thermo Scientific). For chromatographic separation, a mobile phase consisting of acetonitrile and 0.1% formic acid (70:30 (v/v)) was used at an isocratic flow rate of 1 mL/min, along with a Chromolith RP18e column (100 × 4.6 mm, Merck). The column oven temperature was set to 30 °C. Samples were injected in 5 µL volumes. Detection of fipronil, (S)-methoprene, and their major related substances was performed at a wavelength of 245 nm.

During method development, the chromatographic conditions were optimized to achieve complete separation of the target analytes while maintaining a short analysis time and low system back-pressure. In preliminary studies, during method optimization, acetonitrile ratios %80 [18] and %90 [19] mentioned in the literature were evaluated. The selected mobile phase consisting of acetonitrile and 0.1% formic acid (70:30, v/v) provided the best compromise between chromatographic resolution, peak symmetry, analysis

time, and operating pressure among the chromatographic conditions evaluated. This mobile phase enabled complete separation of all analytes within 12 min while maintaining a low operating back-pressure (~13 bar), making it suitable for routine quality control applications.

2.5. Method development

The impact of column dimensions and structures on analyte separation was investigated during method development. Flow rate, temperature, and injection volume were kept constant at 1 mL/min, 30 °C, and 5 µL, respectively. Specifications for the various columns tested are summarized in Table 1. Column performance was compared in terms of system back-pressure (bar), analysis time (time to the last peak), and resolution (R_s , particularly between the fipronil/fipronil sulfone and cis/trans-methoprene pairs).

To justify the selection of the final analytical column, the chromatographic performance of all evaluated stationary phases was systematically compared in terms of system back-pressure, analysis time, retention behaviour, and separation performance. A comparative summary of the tested columns is presented in Table 1. The Chromolith RP-18e monolithic column was selected because it provided complete separation of the target analytes and their major related substances within a short analysis time while operating at substantially

lower back-pressure than the conventional particulate columns. Therefore, this column was selected for subsequent validation studies.

2.6. Method validation

The analytical method was validated for spot-on solutions under the VICH guidelines [23]. The validation parameters and acceptance criteria are shown in Table 2.

Selectivity: Chromatogram results were examined by injecting 5 µL volumes of the following into the HPLC system separately: blank (mobile phase), solvent (acetonitrile), mixed standard working solution (100–120 µg/mL), commercial product placebo, and spiked samples (100–120 µg/mL) [24].

Linearity: Calibration curves were constructed using five concentration levels corresponding to 80%, 90%, 100%, 110%, and 120% of the target concentration. Each concentration level was analysed in triplicate ($n = 15$), and linear regression analysis was performed using the least-squares method based on all individual measurements [24].

LOD and LOQ: These were calculated using Equations 1 and 2, based on the slope (S) of the linearity graph and the standard deviation (σ) of the response obtained from the regression of the linearity data [24,25].

$$\text{LOD} = 3.3 \sigma/S \quad (1)$$

$$\text{LOQ} = 10 \sigma/S \quad (2)$$

Table 2. Validation summary of the fipronil and (S)-methoprene combined products.

Parameter & Acceptance Criteria	Fipronil	Fipronil-Sulfone	Cis-Methoprene	Trans- Methoprene
Selectivity				
No interference	Pass	Pass	Pass	Pass
Peak purity index	≥ 0.99	≥ 0.99	≥ 0.99	≥ 0.99
Linearity				
Regression equation	$y = 0,0795x - 0,0207$	$y = 0,0003x + 0,0009$	$y = 0,0029x - 0,008$	$y = 0,2396x - 0,2407$
$R^2 \geq 0.995$	0,9983	0,9972	0,9952	0,9991
95% CI (slope)	0.0775–0.0814	$3.34 \times 10^{-4} - 3.56 \times 10^{-4}$	0.002747 – 0.002987	0.23395 – 0.24531
LOD (µg/mL)	0.005	0.007	0.013	0.008
LOQ (µg/mL)	0.015	0.021	0.040	0.024
Precision				
%RSD ≤ 2.0 ($n=6$)	0.32	1.26	0.76	0.34
Intermediate precision				
%RSD ≤ 2.0 ($n=12$)	0.52	1.45	1.51	0.67
Accuracy				
(Recovery %, mean $\pm$ SD, $n=3$)	99.08 $\pm$ 0.42	96.87 $\pm$ 1.87	95.68 $\pm$ 0.10	97.14 $\pm$ 1.92
Robustness				
Difference $\leq 2\%$				
Flow (± 0.1 mL/min)	0.94-0.99	0.22-0.29	0.36-1.9	0.61-0.86
ACN ($\pm 1\%$)	0.15-0.23	1.06-1.60	0.19-0.21	0.16-0.22
Temperature ($\pm 2^\circ\text{C}$)	0.09-0.16	0.15-1.14	0.07-0.93	0.19-0.32
System suitability				
Repeatability – retention time $< 1\%$	0.05%	0.08%	0.07%	0.08%
Repeatability – area $< 1\%$	0.36%	1.34%	0.57%	0.35%
Theoretical plates ≥ 2000	3903	3387	9059	9078
Resolution ≥ 2.0	3.2	3.7	2.3	-
Asymmetry= 0.8–1.8	1.6	1.3	1.3	1.3

Note: Data are expressed as mean $\pm$ SD ($n = 3$) for accuracy. Repeatability was assessed from six replicate injections ($n = 6$). Intermediate precision was evaluated on two separate days by two analysts. Calibration curves were constructed using five concentration levels (80–90–100–110–120%), each analysed in triplicate ($n = 15$) using least-squares linear regression. Standard errors of the regression slopes were used to calculate the 95% confidence intervals.

Accuracy: Accuracy was evaluated by recovery studies using spiked placebo samples prepared at 80%, 100%, and 120% of the target concentration. Recovery values were calculated by comparing the measured concentrations with the corresponding theoretical concentrations [24].

Precision: Repeatability was assessed by analysing spiked samples prepared at 100% concentration in six replicate runs. Intermediate precision was evaluated on two separate days using spiked samples prepared at 100% concentration by different analysts.

System suitability: Six injections were performed using a reference standard solution prepared at 100% [26–28].

Robustness: The reference standard solution prepared at 100% was evaluated by analysing it under minor, deliberate changes in method parameters, as summarized in Table 2 [29]. The stability of standard and sample solutions was assessed at 25 °C and 4 °C over 48 h. No significant variation in chromatographic responses was observed (%RSD < 2.0%), indicating that the solutions remained stable during the study period.

2.7. Green analytical chemistry assessment

The environmental impact of the analytical method developed was systematically assessed using the complementary AGREE and GAPI approaches. These tools were selected because they provide comprehensive and standardized evaluations of analytical methods based on the principles of Green Analytical Chemistry, allowing both quantitative and qualitative assessment of environmental performance. Using AGREE (Analytical Greenes Calculator, v.0.5 beta, Gdańsk University of Technology), a quantitative assessment was carried out based on the 12 principles of GAC [21]. Additionally, using complex GAPI (green analytical procedure index, v. 2.0 beta, Gdańsk University of Technology), a qualitative assessment was carried out based on the entire analytical workflow, including sample preparation, reagent toxicity, instrumentation, and waste generation. Each parameter was classified using a colour-coded system (green, yellow, and red) that enables the rapid visualisation of environmental performance [22].

2.8. Statistical analysis

Validation data and sample analysis results were processed using Microsoft Excel® (Microsoft Corporation, Redmond, WA, USA). Linear regression analysis was performed using the least-squares method by plotting analyte concentration against peak area. Regression equations, coefficients of determination (R^2), standard errors (SE), and 95% confidence intervals (CI) for the slope were calculated using the Regression tool in

the Excel Data Analysis ToolPak. LOD and LOQ values were calculated from the standard deviation of the response and the slope of the calibration curve in accordance with VICH recommendations. Validation results are expressed as mean $\pm$ standard deviation (SD).

3. Results

3.1. Method development results

The separation performance of the tested columns and the average system back-pressure results they produced are presented in Table 1. Although the columns, with a particle size of 5 μm and a diameter of 4.6 mm, were able to tolerate a mobile phase flow rate of 1 mL/min, they failed to achieve separation of the two active ingredients and major related substances. System back-pressure was observed at levels of 30–100 bar. The Waters Acquity UPLC BEH C18 column, which has a smaller particle size (1.7 μm), operating pressure exceeded the acceptable limit at a flow rate of 1.0 mL/min.

As shown Fig. 1, the analysis time required for retention of the last peak was generally longer in the 250 $\times$ 4.6 mm, 5 μm columns. However, although the analysis time required for retention of the last peak was short in the 250 $\times$ 4.6 mm, 3 μm phenyl column, complete separation of the fipronil and (S)-methoprene peaks could not be achieved.

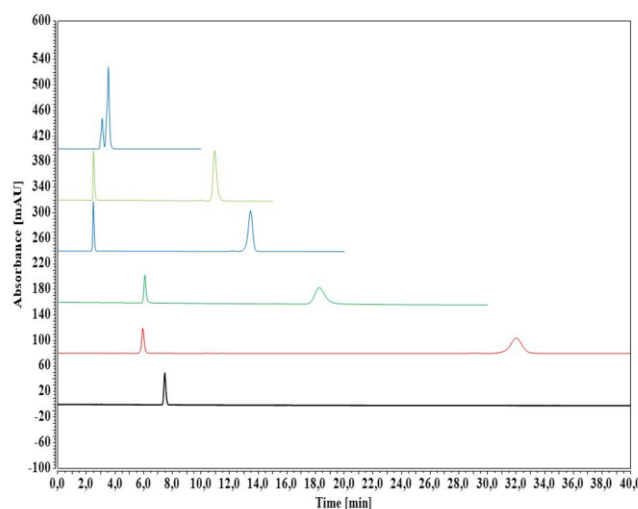


Figure 1. Representative chromatograms obtained during method development using the evaluated stationary phases. The investigated columns included Merck Purospher STAR RP-18e (black line), ACE 5 C18 (red line), Phenomenex Prodigy ODS3 (dark green line), Supelco Discovery C18 (light blue line), Merck Chromolith RP-18e (light green line), and Phenomenex SphereClone Phenyl (dark blue line). For each chromatogram, the first major peak corresponds to fipronil, whereas the second major peak corresponds to (S)-methoprene. Among the evaluated stationary phases, the Merck Chromolith RP-18e monolithic column provided complete separation of fipronil, fipronil sulfone, cis-methoprene, and trans-methoprene within 12 min while maintaining low system back-pressure (12–15 bar). The remaining columns exhibited insufficient chromatographic resolution, inadequate retention of (S)-methoprene, excessive analysis time, or operating limitations under the evaluated chromatographic conditions.

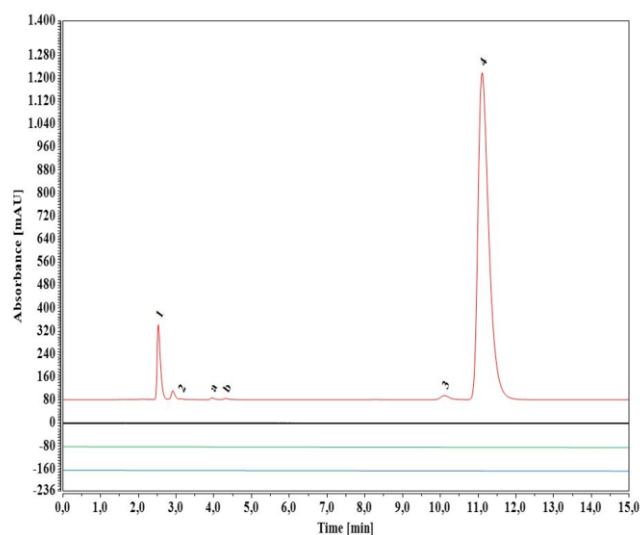


Figure 2. Chromatograms of mobile phase (blue line), diluent (green line), placebo (black line), and spiked sample (red line). 1-Fipronil, 2-fipronil sulfone, 3-cis-methoprene, 4-trans-methoprene, a and b- (S)-methoprene related unknown substances. Detection at 245 nm.

Neither the phenyl column nor the conventional C18 columns provided separation of the active ingredient and their major related substances. On the other hand, among the columns tested, the Merck Purospher Star RP-18e (250 × 4.6 mm, 5 µm) column, which features an end-capped technology, failed to retain (S)-methoprene over a 40-minute analysis time. The monolithic Chromolith RP18e (100 × 4.6 mm) column, featuring end-capped technology, provided separation of the two active ingredients and their major related substances with reasonable resolution values ($R_s = 3.7$ for fipronil/fipronil sulfone and $R_s = 2.3$ for cis-/trans-methoprene). Furthermore, the average system back-pressure was approximately 13 bar, allowing complete separation of all analytes within 12 min. Based on these results, this column was selected for subsequent validation studies.

As summarized in Table 1, none of the conventional particulate columns simultaneously fulfilled the chromatographic requirements for rapid analysis, adequate separation of the critical analyte pairs, and acceptable system back-pressure. Conventional C18 and phenyl columns either failed to resolve the related substances or produced excessive analysis times, whereas the end-capped particulate RP-18e column did not provide adequate retention of (S)-methoprene within the evaluated chromatographic run time. In contrast, the Chromolith RP-18e monolithic column achieved baseline separation of all analytes with satisfactory resolution values and the lowest operating back-pressure, supporting its selection for subsequent validation studies.

3.2. Method validation results

The analytical method developed was successfully validated for the simultaneous determination of fipronil and (S)-methoprene in veterinary spot-on formulations. As shown in Fig. 2, no interference from the mobile phase, solvent, or blank was detected at the retention times of the active ingredients and their major related substances, confirming the selectivity of the method. Peak purity indices obtained from DAD detection were higher than 0.99 for all analytes. Representative PDA peak purity plots are presented in Fig. 3, demonstrating spectral homogeneity across each chromatographic peak and further supporting the specificity of the method.

The sensitivity of the method enabled the detection of major related substances at levels below 0.05% [24]. For both active ingredients and related substances, the coefficient of determination (R^2) was ≥ 0.995 , repeatability was below 2.0% RSD, and recovery values ranged from 95.68% to 99.07%, fulfilling the VICH acceptance criteria [24]. Suitable values were obtained for the system suitability parameters [25,27,28]. The robustness of the analytical method was successfully tested [27].

3.3. Results of green analytical chemistry assessment

As shown in Fig. 4, the AGREE assessment of the proposed HPLC-DAD method demonstrated an overall greenness score of 0.78. The twelve segments of the circular pictogram represent the twelve principles of Green Analytical Chemistry, while the colour coding (green, yellow, and red) reflects the environmental performance of each individual parameter.

As shown in Fig. 5, the ComplexGAPI pictogram provided a complementary qualitative assessment of the analytical workflow. The sample preparation stage (parameters 1–5) was entirely green because the direct dilution procedure eliminated the need for hazardous extraction solvents. The reagent and waste management sections (parameters 6–11 and 15) contained yellow zones due to the use of acetonitrile, whereas the instrumentation section (parameters 12–14) remained green, reflecting the low operating back-pressure (~13 bar) of the monolithic column. Furthermore, the calculated E-factor was approximately 10.5.

3.4. Results of product analysis

The levels of active and related substances were quantified by comparing the peak areas of the analytes in the chromatograms obtained from the reference standard solutions with those obtained from the product samples. The analytical results are summarized in Table 3.

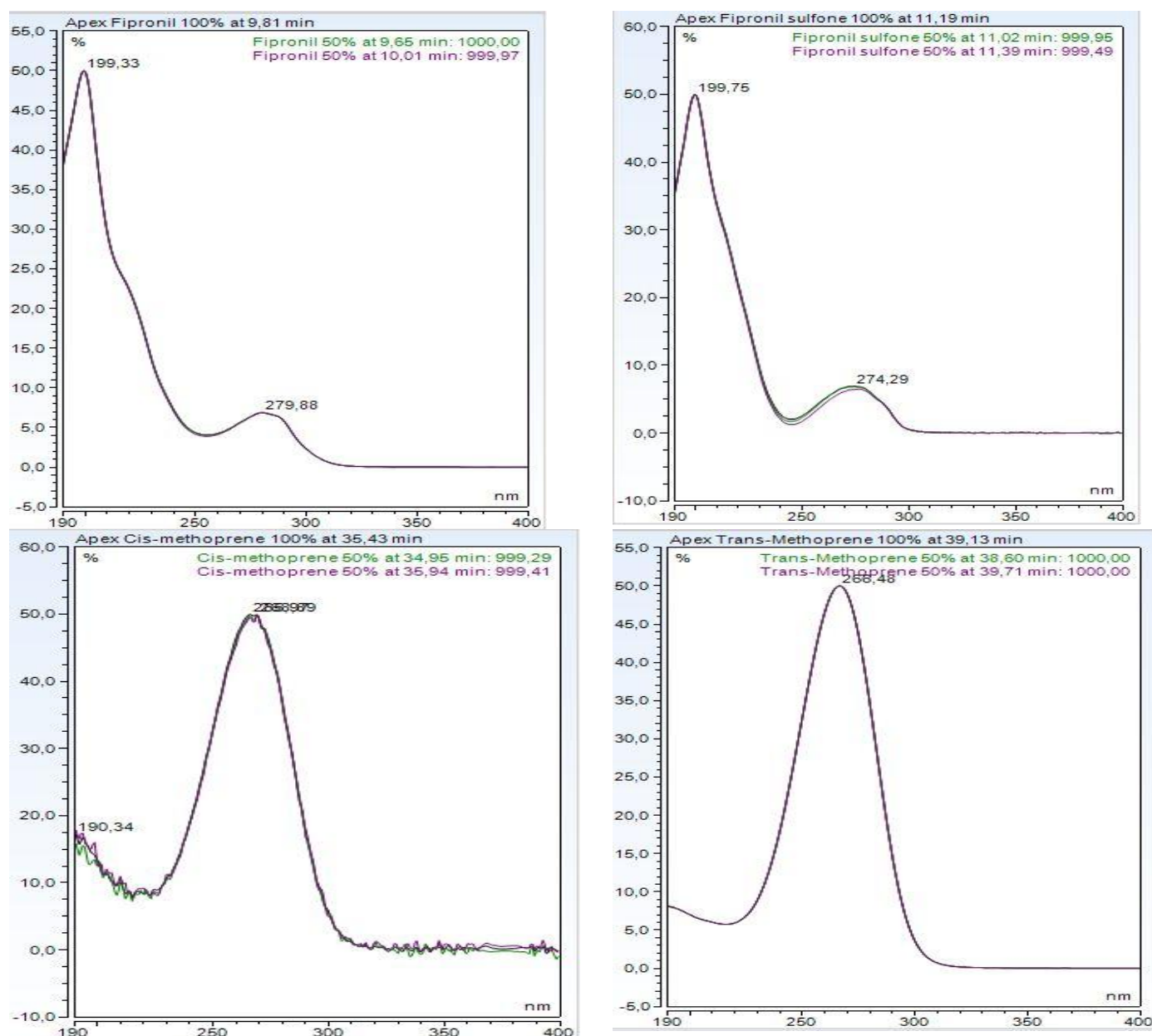


Figure 3. Representative peak purity reports obtained by DAD spectral comparison for fipronil, fipronil sulfone, cis-methoprene, and trans-methoprene. Spectral overlay at the upslope, apex, and downslope of each chromatographic peak demonstrated peak purity indices greater than 0.99, confirming the absence of co-eluting compounds

Table 3. Analysis results of fipronil and (S)-methoprene combined products.

Product ID	Fipronil assay (% ± SD)	Fipronil/ Fipronil sulfone ratio (% ± SD)	(S)-methoprene assay (% ± SD)	Trans/cis-methoprene ratio (% ± SD)	Assessment
P1	93.97 ± 0.76	0.67 ± 0.44	94.74 ± 0.56	1.77 ± 0.64	Compliant
P2	91.10 ± 0.35	0.69 ± 0.32	91.86 ± 0.45	1.15 ± 0.22	Compliant
P3	91.34 ± 0.24	0.46 ± 0.12	92.10 ± 0.61	1.60 ± 1.12	Compliant
P4	93.44 ± 0.56	0.96 ± 0.30	94.21 ± 0.76	1.86 ± 0.55	Compliant
P5	96.82 ± 0.62	0.88 ± 0.40	97.60 ± 0.98	0.96 ± 0.40	Compliant
P6	97.96 ± 0.51	0.54 ± 0.18	99.12 ± 0.72	1.90 ± 0.38	Compliant
P7	101.02 ± 1.23	0.71 ± 0.24	98.23 ± 0.54	1.10 ± 0.41	Compliant
P8	100.24 ± 1.02	0.68 ± 0.19	96.54 ± 0.77	0.88 ± 0.50	Compliant
P9	97.33 ± 0.76	0.56 ± 0.20	99.05 ± 1.02	0.50 ± 0.14	Compliant
P10	97.45 ± 0.54	0.74 ± 0.33	99.20 ± 1.10	1.70 ± 0.25	Compliant

SD (standard deviation). Fipronil and (S)-methoprene active substances shelf life limits ±10%. Fipronil sulfone limit < 2%, cis-methoprene limit < 6%.

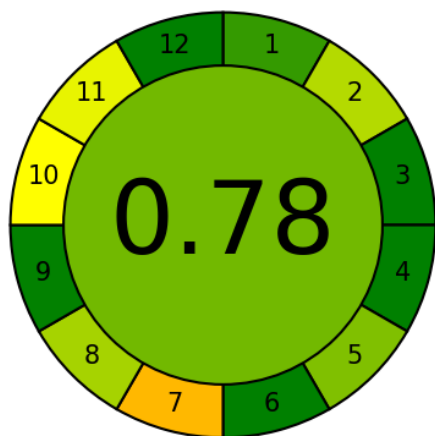


Figure 4. In the green analytical evaluation of the proposed HPLC-DAD method using the AGREE metric.

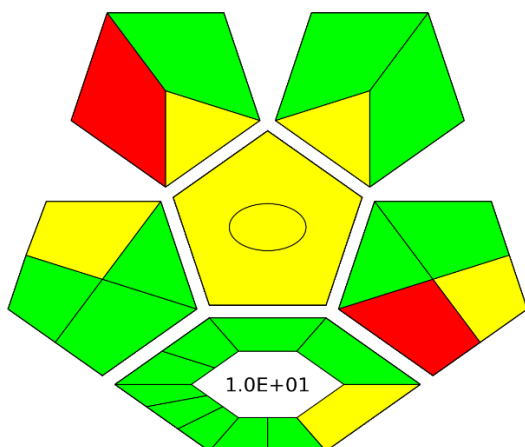


Figure 5. Evaluation of GAC using the complex GAPI pictogram of the proposed HPLC method.

Related substance identification was carried out by analyzing the UV absorption spectra in the range of 210–400 nm. The fipronil and fipronil sulfone peaks were detected at retention times of 2.5 and 3.1 min, respectively. Their maximum UV absorption wavelengths in acetonitrile were 280 and 274 nm, respectively, and both peaks exhibited purity index values greater than 0.99.

Similarly, the cis- and trans-methoprene peaks were observed at 10.1 and 11.1 min, respectively, with both isomers exhibiting a maximum UV absorption at 260 nm. The peak purity indices of both isomers were also greater than 0.99. In addition, two minor peaks associated with the methoprene region were detected at levels below 1%. Since certified reference standards were not available, these peaks could not be structurally identified. Their UV spectra differed from those of cis- and trans-methoprene, and no interference with quantification of the target analytes was observed. Representative DAD peak purity reports are presented in Fig. 3. Spectral

overlay at the upslope, apex, and downslope of each chromatographic peak confirmed peak purity indices greater than 0.99, supporting the selectivity of the proposed method.

A detection wavelength of 245 nm was selected to enable the simultaneous determination of all analytes with adequate sensitivity. All peak resolutions were found to be within the pharmacopoeial limits [8]. When the areas of all detected impurities were normalized relative to the fipronil peak area, the total impurity level did not exceed 3.0%.

The analyzed product samples were found to comply with the shelf-life specifications and relevant guidelines [4,5,7] in terms of active substance content, impurity limits for fipronil sulfone and cis-methoprene (3.5% and 6%, respectively), and total detectable impurity levels.

4. Discussion

In this study, a simple, rapid, and isocratic reverse-phase HPLC-DAD method utilising an end-capped monolithic column was successfully developed and validated for the simultaneous determination of fipronil, (S)-methoprene, and their major related substances (fipronil sulfone and cis-methoprene) in veterinary medicinal products. The main contribution of the proposed method is the combination of an end-capped monolithic silica column with isocratic elution, enabling simultaneous determination of the active substances and their major related substances while also providing a systematic evaluation of the method using Green Analytical Chemistry metrics. Compared with previously reported HPLC methods, the proposed approach combines rapid isocratic separation, simultaneous determination of the active substances and their major related substances, and systematic evaluation using Green Analytical Chemistry metrics within a single validated analytical procedure.

Compared to the back-pressure range of 30–100 bar observed in conventional C18 particle columns under the same chromatographic conditions, the monolithic column provided a significantly lower back-pressure (~13 bar) [20]. The bimodal pore structure of monolithic columns (macro- and mesopores) minimizes mass transfer resistance, even at high flow rates, thereby enabling efficient separation within a short analysis time of 12 min in accordance with European Pharmacopoeia standards [8]. The obtained resolution values, acceptable peak symmetry (0.8–1.8), and high peak purity index (> 0.99) collectively demonstrate the robustness and reliability of the developed method. In contrast, most of the conventional particle columns tested (5 μ m and 3 μ m) failed to provide sufficient resolution of the analytes or resulted in long analysis times of up to 32 minutes.

The systematic comparison of seven stationary phases demonstrated that the final column selection was based not only on chromatographic resolution but also on analysis time and operating back-pressure, thereby providing a balanced approach for routine quality control applications.

Although UPLC-based methods reported in the literature offer shorter run times, they are associated with high equipment costs and risks of matrix-induced clogging [8,15]. Similarly, many existing HPLC methods are limited by long analysis times, the complexity of gradient elution, or limited data on impurity profiling [16–19]. A comparative summary of the proposed method and previously reported chromatographic methods is presented in Table 4. As shown in Table 4, the analytical performance obtained in the present study is comparable with previously reported methods while additionally providing low operating back-pressure, rapid isocratic separation, and integrated evaluation using AGREE and ComplexGAPI metrics. The LOD and LOQ values obtained in our study further demonstrate that the method's sensitivity is consistent with the literature [15,17] and that it offers high reliability for quality control processes [26].

In terms of the method's environmental sustainability, the score of 0.78 obtained from the AGREE analysis indicates good compliance with GAC principles [21]. The complex GAPI analysis demonstrates that by simplifying sample preparation to a single-step dilution, we have minimized chemical exposure and procedural complexity. Although acetonitrile was used as the organic solvent, the total solvent consumption was approximately 12 mL per analysis, which is lower than that reported for many conventional HPLC methods requiring longer run times and post-run column equilibration [8,16–19]. Furthermore, the use of isocratic elution eliminated the need for gradient re-equilibration, further improving the efficiency of the analytical procedure. The low E-factor further supports the improved environmental performance of the proposed method, making it suitable for sustainable quality control in veterinary pharmaceutical analysis [22]. The simplified sample preparation procedure, based solely on dilution without extraction or derivatisation, further contributed to reducing chemical consumption and analytical complexity. The low system back-pressure provided by the monolithic column may also contribute to reduced energy consumption while extending column lifetime,

Table 4. Comparison of the proposed method with previously reported methods for the determination of fipronil, (S)-methoprene, and related substances.

Parameter	[8]	[15]	[16]	[17]	[18]	[19]	This study
Column characteristics	Particulate, 150 × 4.6 mm, 1.8 µm	Particulate, 50 × 2.1mm, 2.0 µm	Particulate, 250 × 4.6 mm, 5.0 µm	Particulate 150×4.6 mm, 2.7 µm A: 10 mM potassium phosphate buffer/1,4- Dioxane (98:2 v/v) B: EtOH/MeOH/ACN/IPA (86:4:6:4 v/v/v/v) 0.6 mL/min, gradient	Particulate 150×4.6 mm, 5.0 µm	Particulate, 100×4.6 mm, 5.0 µm	Monolithic silica 100×4.6 mm
Mobile phase	THF/MeOH/H ₂ O /ACN (0.5:30:30:40 v/v/v/v) 1.0 mL/min, isocratic	0.1% H ₃ PO ₄ /ACN/MeOH (50:50 v/v) 0.4 mL/min, gradient	H ₂ O/MeOH/ACN (40:25:35 v/v/v) 1.0 mL/min.-isocratic		ACN/H ₂ O (90:10 v/v) 1.0 mL/min, isocratic	ACN/H ₂ O (80:20 v/v) 1.5 mL/min, isocratic	ACN/0.1% formic acid (70:30 v/v) 1.0 mL/min, isocratic
Analysis time	~12 min	6 min	22 min	70 min	10 min	15 min	12 min
System back-pressure	NR	NR	NR	NR	NR	NR	~13 bar
Simultaneous determination of fipronil & (S)-methoprene	No	No	No	Yes	Yes	Yes	Yes
Related substance determination capability	Fipronil	Fipronil	Fipronil	Fipronil + (S)-methoprene	-	-	Fipronil + (S)-methoprene
LOD-LOQ	NR (EP monograph)	LOQ 0.05%	LOD 0.1%	LOD 0.1% LOQ 0.3%	NR	Fipronil: LOD 0.57 µg/mL LOQ 1.69 µg/mL (S)-methoprene: LOD 0.21 µg/mL LOQ 0.65 µg/mL	LOQ 0.05%
Green analytical assessment	No	No	No	No	No	No	Yes (AGREE, ComplexGAPI)

Abbreviations: ACN, acetonitrile; MeOH, methanol; IPA, isopropanol; EtOH, ethanol; H₂O, water; NR, not reported; EP, European Pharmacopoeia; Tetrahydrofuran, THF.

thereby providing an additional practical and economic advantage for routine quality control laboratories.

Despite some of its advantages, the study has structural and operational limitations. The use of acetonitrile as the primary organic solvent poses an environmental constraint; in the future, this solvent could be replaced with greener alternatives such as ethanol, provided that comparable chromatographic performance can be achieved. Furthermore, the method presented is specifically optimised for spot-on (liquid) formulations; the use of LC-MS or additional validation steps may be required for more complex biological matrices or solid dosage forms [15,17]. The two minor unidentified peaks observed in the methoprene region were present at low levels (<1%) and did not interfere with the quantification of the target analytes. Structural identification of these compounds would require complementary techniques such as LC-MS and was therefore considered beyond the scope of the present study. Forced degradation studies were beyond the scope of the present study and may be considered in future investigations aimed at developing a stability-indicating analytical method. Finally, this study is a pilot-scale evaluation conducted with a limited number of samples.

Consequently, the proposed method strikes a positive balance between analytical performance and environmental impact, offering a robust option for routine quality control laboratories seeking low-cost, rapid, and sustainable solutions. The successful analysis of commercial products (P1–P10) further demonstrates the applicability of the proposed method for routine quality control, post-marketing surveillance, and regulatory control activities [27,28].

5. Conclusion

In conclusion, this isocratic RP-HPLC-DAD method utilising an end-capped monolithic column represents a practical, efficient, and environmentally conscious alternative for the simultaneous determination of fipronil, (S)-methoprene, and their major related substances in spot-on formulations. The AGREE and ComplexGAPI assessments conducted as part of this study demonstrated good compliance of the proposed method with Green Analytical Chemistry principles, showing that high analytical performance can be achieved while reducing the environmental impact of the analytical procedure. The method's simplicity, low cost, rapid analysis, and sensitivity for detecting major related substances make it a suitable option, particularly for quality control laboratories with high sample throughput. Overall, the proposed method provides a balanced analytical approach from both technical and

environmental perspectives and represents a practical tool for routine pharmaceutical quality control and post-marketing surveillance of veterinary medicinal products.

Conflict of interest

The author declares that there are no conflicts of interest regarding the publication of this paper.

Author contributions

The author confirms sole responsibility for the following: study conception and design, data collection, analysis and interpretation of results, and manuscript preparation.

AI statement

Generative AI was used only for English language editing and grammar checking. All scientific content, data interpretation, and conclusions were developed, verified, and approved by the authors, who take full responsibility for the manuscript.

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