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Development and Single Laboratory Validation of Ultra-Fast Liquid Chromatography Method for Quantification of Bispyribac sodium and Difenthiuronin in Bispyribac sodium Suspension Concentrate (SC) and Difenthiuron Wettable Powder (WP) Formulations

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ABSTRACT

Background: *Bispyribac sodium_is the organic sodium salt of bispyribac. It is used as a broad-spectrum post-emergent herbicide for the control of grasses, sedges and broadleaf weeds in rice crops. Diafenthiuron is an aromatic ether that an agricultural pro-insecticide that is used to control mites, aphids, and whitefly in cotton. There is no validated method for quantification of Bispyribac sodium and Difenthiuron in pesticide formulations.*

Objective: *This study aimed to develop a simple, specific, accurate, precise and reliable UFLC method for the quantification of Bispyribac sodium and Difenthiuron in their pesticide formulations.*

Methods: *Bispyribac sodium and Difenthiuron were quantified by using Shimadzu, packed with C18 (250 mm × 4.6 mm, 5 µm) column. A mixture of acetonitrile: water (50:50 and 70:30 v/v) was used as a mobile phase for Bispyribac sodium and Difenthiuron respectively. The column oven temperature was maintained at 25°C throughout the analysis. The flow rate was maintained at 1.2 and 1.0 ml/min and detection at 254 and 225 nm for Bispyribac sodium and Difenthiuron respectively was carried out with injection volume of 5 and 2µl. The % RSD values for the precision study were <1.19 and <1.60 for Bispyribac sodium and Difenthiuron respectively. The analytical method was validated according to the guidelines described in Collaborative International Pesticides Analytical Council (CIPAC).*

Results: *The UFLC method was able to quantify Bispyribac sodium and Difenthiuronin in their formulations by gradient elution within 15 and 20 min respectively. % RSD in retention times (R.T.) of Bispyribac sodium and Diafenthiuron was found to be 0.060 and 0.218 respectively. % RSD in the peak area of Bispyribac sodium and Diafenthiuron was found to be 0.218 and 0.403. The % recovery of Bispyribac sodium and Difenthiuron was found to be 101.14 and 99.38%.* ***Conclusion:*** *The UFLC method for assay of Bispyribac sodium and Difenthiuron was successfully developed, validated, and demonstrated to be accurate, precise and specific.*

Keywords: *Bispyribac sodium and Diafenthiuron res, CIPAC - Collaborative International Pesticides Analytical council, Horwitz equation, Method development, Method validation, Uncertainty in measurements, UFLC, Pesticide analysis.*

INTRODUCTION

Bispyribac sodium, is sodium; 2, 6- bis [(4, 6- dimethoxy pyrimidin- 2- yl) oxy] benzoate. It is used as a broad-spectrum post-emergent herbicide for the control of grasses, sedges and broadleaf weeds in rice crops. It has a role as an herbicide, an agrochemical and an environmental contaminant. It contains a bispyribac. Bispyribac sodium (Figure 1) is a systemic herbicide belonging to pyrimidinyl carboxy class. It inhibits the enzyme, acetolactate synthase which is necessary for the growth of plants. The herbicide also inhibits the synthesis of branch-chain amino acid viz. valine, leucine, and isoleucine. Bispyribac sodium has low toxicity to birds, earthworms, bees, aquatic invertebrates and fish [1]. Diafenthiuron (1- tert- butyl- 3- (2, 6-diisopropyl thiourea)) is a insecticide, which is the only non organo fluorine benzyl urea compound having novel insecticide and acaricide activity acting selectively on group of insects/mites [2] by inhibiting or enhancing biochemical sites such as respiration [3]. Diafenthiuron is reported to be effective against whiteflies and leaf hoppers in brinjal [4, 5], whiteflies, aphids and thrips in tomatoes [6, 7, 8], thrips and mites in chilies and tea [9, 10], and all sucking pests in cotton [11, 12]. Diafenthiuron is effective against diamond back moth (DBM) [13, 14], shoot and capsule borer, and thrips in cardamom [15, 16] and it is gaining momentum now in cardamom plantations.

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A method is described for the determination of the bispyribac sodium in surface water. The method involves extraction in solid phase and quantification by high-performance liquid chromatography with diode array detection (HPLC- DAD) [17]. An analytical method employing HPLC with a diode array detector was developed to determine bispyribac sodium residues in rice. Bispyribac sodium residues in rice were further confirmed by LC–MS [18]. The residues of Bispyribac sodium 10 % SC on rice crop samples were analyzed for Bispyribac sodium content by a validated HPLC method at the minimum detectable concentration of 0.01 ppm [19]. A simple, rapid, sensitive, and suitable method is developed for the simultaneous determination of bispyribac sodium in rice by liquid chromatography-tandem mass spectrometry (LC-MS/MS) coupled with modified QuEChERS. The analytes were separated on a ZORBAX SB C_{18} column through a gradient elution using 0.1% (v/v) aqueous formic acid aqueous containing 5 mmol/L ammonium acetate and acetonitrile as mobile phases [20].

Two methods were developed to determine Diafenthiuron in coconut water. The first procedure involves solid-phase extraction using Sep-Pak Vac C_{18} disposable cartridges with methanol for elution. Isocratic analysis was carried out by means of high-performance liquid chromatography with ultraviolet detection at 254 nm. The other procedure is based on liquid–liquid extraction with hexane–dichloromethane (1:1, v/v), followed by gas chromatographic analysis with effluent splitting to electron-capture detection [21]. Another study was carried out to analyze the effect of traditional food processing on the reduction of diafenthiuron residues in cauliflower through GC-μECD and HPLC [22]. A simple analytical method based on QuEChERs was established for diafenthiuron residues in packhoi and soil. The residue levels and dissipation rates of diafenthiuron in pakhoi and soil were detected by HPLC–MS. And ultrasonic extraction was employed in the study to improve extraction effectiveness [23]. A dispersive liquid–liquid microextraction (DLLME) method using *in situ* halide exchange reaction to form ionic liquid (IL) extraction phase was developed to determine diafenthiuron in water samples. The pre-concentration procedure is followed by high-performance liquid chromatography and variable wavelength detectors (VWD) [24, 25]. These analytical methods are applicable only for pesticide residue analysis. These are not applicable to formulation analysis.

To the best of our knowledge, there is no reported method for quantification of Bispyribac sodium and Difenthiuron in pesticide formulations. This study aimed to develop a simple, specific and reliable UFLC method for the quantification of Bispyribac sodium and Difenthiuron in their pesticide formulations. The active component analysis of Bispyribac sodium and Difenthiuronin in pesticide Formulations is required for the regular quality control analysis of production samples, environmental samples and identification of spurious pesticides formulation products in the market. Thus, efforts were made to develop fast, selective and sensitive method for quantification of Bispyribac sodium and Difenthiuron in their pesticide formulation using ultra-fast liquid chromatographic method. In the current work developed a simple, reliable and reproducible, stability indicating UFLC method which was duly validated by statistical parameters precision, accuracy-recovery, and linearity following the recommended guidelines of CIPAC. Uncertainties in measurements were also calculated for each active ingredient.

EXPERIMENTAL PROCEDUDRE

Materials: Certified Reference materials (CRM) of Bispyribac sodium and Difenthiuron were procured from Sigma Aldrich. The technical grade materials of the above active ingredients were obtained from the market. The analytical standards were prepared by purification of these technical grade materials. The analytical standards were qualified against CRMs and calculated purity was found as for Bispyribac sodium- 98.27% and Difenthiuron- 97.46%. These standards used for further analysis. Bispyribac sodium 10% SC and Difenthiuron 50% WP were procured from market. HPLC-grade acetonitrile was purchased from Fischer Scientific, Mumbai (India). Mili-Q (Millipore India Pvt. Ltd) system used to obtain HPLC grade water.

Instrumentation: The UFLC system used to perform the development and validation of this quantification method is of Shimadzu UFLC comprised of a binary solvent pump, Photo Diode array detector and auto sampler with lab solutions software.

Mobile phase preparation: The mobile phase consist of Mobile phase A –Acetonitrile and Mobile phase B – Water in 50:50 and 70:30(v/v) ratio for Bispyribac sodium and Difenthiuron respectively. Mobile phase- B was filtered through a 0.45 μm nylon membrane (Millipore Pvt. Ltd, Bangalore, India) and degassed in an ultrasonic bath.

Diluent preparation: Mobile phase used as diluent.

Standard Preparation: The Standard stock solution was prepared in 50 ml volumetric flask by dissolving 50.88 mg of Bispyribac sodium (98.27%) and 51.30 mg of Difenthiuron (97.46%) standard in 10 ml of diluent. This solution then sonicated for 10 minutes and diluted to volume with diluent. This standard solution contains 1 mg/ml of Bispyribac sodium and Difenthiuron respectively.

Sample Preparation: The sample solution was prepared by taking about 500 and 100mg of Bispyribac sodium 10% SC and Difenthiuron 50% WP respectively in 50 ml volumetric flask and about 10 ml of respective diluent was added and sonicated for 10 minutes with intermittent shaking. The content was brought back to ambient temperature and diluted to volume with diluent. The sample was filtered through 0.45μm nylon syringe filter.

Chromatographic condition: The method involves use of Shimadzu- C18 column with length of 250 mm, internal diameter 4.6 mm and 5μm particle size of stationary phase. The column oven temperature was maintained at 25°C throughout the analysis. Different compositions of the mobile phase are tried in isocratic mode. Mobile Phase-A: Mobile Phase-B Acetonitrile: Water (50:50 and 70:30 (v/v) ratio for Bispyribac sodium and Difenthiuron respectively) was selected which gave good resolution. The flow rate was maintained at 1.2 and 1.0 ml/min and detection at 254 and 225 nm for Bispyribac sodium and Difenthiuron respectively was carried out with injection volumes of 5 and 2μl.

Statistical Analysis

Precision: The Precision was evaluated by repeatability. Each level of precision was investigated by five replicate injections of a standard solution of Bispyribac sodium and Difenthiuron with a concentration about 1 mg/ml each and 5 different preparations of same sample. Table 3 shows acceptable % RSD values calculated by the modified Horwitz equation. The Horwitz equation for acceptable repeatability was defined by

Horwitz from a practical consideration of a number of collaborative studies done by AOAC over many years.

The equation is: $\%\ RSD_R = 2^{(1-0.5\log C)}$

Where $\%RSD_R$ is the inter-laboratory CV and C is the concentration of analyte in the sample as a decimal fraction

Horwitz noted that values for RSDr (the repeatability CV) were usually between half and two-thirds that of RSD_R. For this reason, repeatability acceptabilities are proposed as the Horwitz values for RSD_R x 0.67.

$RSD = <2^{(1-0.5\log C)} \times 0.67$

Accuracy and recovery: The accuracy of the procedure was determined by the examination of a number of samples containing a known quantity of the analyte. The market sample of pesticide is spiked with a known quantity of analyte (corresponding to the quantity demanded by the method) is added. The analyte added was a technical active ingredient of known purity. The whole sample was analysed to eliminate sampling error. Four recovery studies were done, following exactly the proposed procedure. The results were treated as follows:-

Calculated the mean % recovery for the synthetic formulation as follows:

$$\text{Mean \% recovery} = \frac{\text{Mean \% Content determined} \times 100}{\text{Theoretical \% Content}}$$

This % recovery should be within the following ranges:

% Active (nominal)	**Mean % recovery**
>10	98.0 - 102.0
1 - 10	97.0 - 103.0
<1	95.0 - 105.0

Accuracy (% Recovery) of the analytical method was determined at four concentration levels by spiking 28.34% and 17.92% of Bispyribac sodium and Difenthiuron respectively of pure actives in the sample. The theoretical content of Bispyribac sodium and Difenthiuron after spiking in sample was 8.92% and 59.246% respectively. The accuracy was calculated as % of the recovery.

Uncertainty in measurement (U): Uncertainty of method was measured through the data of uncertainty due to Repeatability, Calibration uncertainty of equipment or glassware, Readability of equipment, CRM purity of concentration, Linearity of the calibration curve and Recovery of the analyte [26].

Initial analysis of sample: Sample was analyzed and results were tabulated in table 1.

Calculation:

Active content (% m/v) for Bispyribac sodium / Difenthiuron

$$= \left(\frac{\text{Area of sample}}{\text{Area of standard}}\right) \times \left(\frac{\text{Weight of standard}}{\text{Weight of sample}}\right) \times (\text{purity \%}) \times (1.0)$$

RESULTS AND DISCUSSION

Development and optimization of UFLC Method: In the present work, an analytical method based on UFLC using PDA detector has been developed and validated for the quantification of Bispyribac sodium and Difenthiuron in pesticide formulation. The analytical conditions were selected, keeping in mind the different chemical nature of Bispyribac sodium and Difenthiuron. The column selection has been done on the basis of back pressure, resolution, peak shape and day to day reproducibility of retention time. Shimadzu C18 (250 mm x 4.6 mm, 5µm particle size) column was found to be giving satisfactory results. The selection of the mobile phase is based on the chemical structure of Bispyribac sodium and Difenthiuron. Considerably good results were obtained with water as mobile phase-B. For the selection of organic constituents of mobile phase-A, acetonitrile was chosen to reduce the longer retention time and to attain good peak shape. Finally the mobile phase composition consists of in Mobile phase-A (Acetonitrile): Mobile phase-B (Water) in 50:50 and 70:30 (v/v) ratios for Bispyribac sodium and Difenthiuron respectively. The optimized proportion of mobile phase has shown good resolution for Bispyribac sodium and Difenthiuron.

Specificity: The specificity of the method was determined by injecting mobile phase blank, between the peaks of active ingredients in standard, sample as well as in mobile phase blank. Bispyribac sodium standard, Difenthiuron standard and sample solution. Since there was no interference also peak purity was found satisfactory. Refer figure 3-8. *System Suitability:* System suitability is integral part of method validation. % RSD of retention times (R.T.) of five replicate injections of standard solution of Bispyribac sodium and Diafenthiuron was found to be 0.060 and 0.218 respectively, which was less than the upper limit of 1.0. % RSD of peak area of five replicate injections of standard solution of Bispyribac sodium and Diafenthiuron was found to be 0.218 and 0.403, which was less than the upper limit of 1.0.(Refer Table II).

Precision: The Precision was evaluated by repeatability. Each level of precision was investigated by five replicate injections of a standard solution of Bispyribac sodium and Difenthiuron with concentration about 1 mg/ml each and 5 different preparations of same sample. Acceptable % RSD values calculated by modified horwitz equation of Bispyribac sodium and Diafenthiuron were found to be 1.90 and 1.49 respectively. Table III shows acceptable % RSD values calculated by the modified Horwitz equation.

The equation is: $\%\ RSD_R = 2^{(1-0.5\log C)}$

Where $\%RSD_R$ is the inter-laboratory CV and C is the concentration of analyte in the sample as a decimal fraction

So for a 50% sample (e.g. a 500g/kg WP), C = 0.5 Log C = -0.3010

So, $RSD_R = 2^{(1-(0.5x-0.3010))} = 2^{1.1505} = 2.22\ \%$

Horwitz noted that values for RSDr (the repeatability CV) were usually between half and two-thirds that of RSDR. For this reason, repeatability acceptabilities are proposed as the Horwitz values for RSDR x 0.67.

$RSD = <2^{(1-0.5\log C)} \times 0.67$

For the values above, this gives the following:

% Analyte	Horwitz RSDR	Proposed acceptable RSDr
50	2.22	1.49

The results of the precision study were expressed as % RSD and were tabulated in Table IV. The % RSD of precision study of five replicate injections of a standard solution of Bispyribac sodium and Diafenthiuron was found to be 0.40 and 1.32 respectively, which was less than the upper limit of calculated % RSD 1.90 and 1.49 of Bispyribac sodium and Diafenthiuron respectively.

Linearity: The linearity was evaluated by measuring 5 different concentration levels of standard solution of Bispyribac sodium and Difenthiuron. The linearity study was done in range of 100-474 and 400- 1260 PPM for Bispyribac sodium and Difenthiuron respectively. The linearity curve plotted the concentration of the standard (PPM) against mean peak areas and the correlation

coefficient value was computed. The correlation coefficient value was found to be 0.997 and 0.992 for Bispyribac sodium and Difenthiuron respectively which was more than the recommended level of minimum 0.99. The summary of the parameters were shown in Table V.

Accuracy and recovery: Accuracy (% Recovery) of analytical method was determined at four concentration levels by spiking 28.34% and 17.92% of Bispyribac sodium and Difenthiuron respectively of pure actives in sample. The theoretical content of Bispyribac sodium and Difenthiuron after spiking in sample was 8.92% and 59.246% respectively. The Bispyribac sodium and Difenthiuron content determined or obtained after spiking in the sample were 9.03% and 58.88% respectively. The % RSD of Bispyribac sodium and Difenthiuron was found to be 0.3967 and 0.215 which is less than the maximum limit of 1.90 and 1.49 of % RSD calculated. The accuracy was calculated as % of recovery. The % recovery of Bispyribac sodium and Difenthiuron was found to be 101.14 and 99.38% respectively, which is in the recommended range of 97.0 - 103.0 and 98.0 - 102.0 % for Bispyribac sodium and Difenthiuron respectively. The results were tabulated in Table VI.

Uncertainty in measurement (U): The combined relative uncertainty (Uc) of Bispyribac sodium and Difenthiuron was found to be 0.004667 and 0.007410. The expanded uncertainty (U) of Bispyribac sodium and Difenthiuron was found to be 0.064800 and 0.740960 (% m/v). Refer Table VII

CONCLUSION

A simple, specific and reliable UFLC method has been developed for the quantification of Bispyribac sodium and Difenthiuron their pesticide formulation. The method validation study showed that the method is specific, linear, accurate, and easily reproducible. This method is also useful for quantification of Bispyribac sodium and Difenthiuronin their single or combination formulated products with different strengths and different formulation types. This method can also be useful for analysis of environmental samples (soil, water), and agricultural products for pesticide residue analysis of same actives but required additional extraction procedure. Hence developed method can be adapted to regular quality control analysis of production samples and environmental samples.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Table 1.Results of initial analysis

Sr. No	Ingredients	Active Ingredient content (A.I)	Expiration date
		% m/v	
1.	Bispyribac sodium	6.95	23/05/2023
2.	Difenthiuron	50.20	09/03/2023

Table 2.System Suitability of standard solution

Parameters	Results		Limits
	Bispyribac sodium	Diafenthiuron	
% RSD of retention time	0.060	0.146	< 1.0
% RSD of peak area	0.218	0.403	< 1.0

Table 3.Acceptable % RSD values calculated by modified Horwitz Equation

Sr. no.	Compound	% Analyte (m/v)	Analyte Ratio (C)	% RSD (calc.)
1.	Bispyribac sodium	10	0.10	1.90
2.	Difenthiuron	50	0.50	1.49

Table 4.Results of Precision studies

	Bispyribac sodium	Difenthiuron
Mean (% m/v)	6.95	50.20
% RSD	0.40	1.32

Table 5.Linearity study

	Bispyribac sodium	Difenthiuron
Linearity Range (PPM)	100- 474	400- 1260
Correlation Coefficient (R^2)	0.997	0.992
Slope (m)	1372	22397
Y-intercept (C)	17443	91222

Table 6.Results of accuracy study

Components	Level	Theoretical content of pesticide (%)	Pesticide content determined or obtained (%)	% Mean Recovery	% RSD
Bispyribac sodium	28.34%	8.92	9.03	101.14	0.3967
Difenthiuron	17.92%	59.246	58.88	99.38	1.599

Table 7.Calculated Combined and Expanded Uncertainty

Components	Mean Value (% m/v) (n=20)	Combined Relative Uncertainty (Uc)	Expanded Uncertainty (U) (% m/v)
Bispyribac sodium	6.95	0.004667	0.064800
Difenthiuron	50.20	0.007410	0.740960

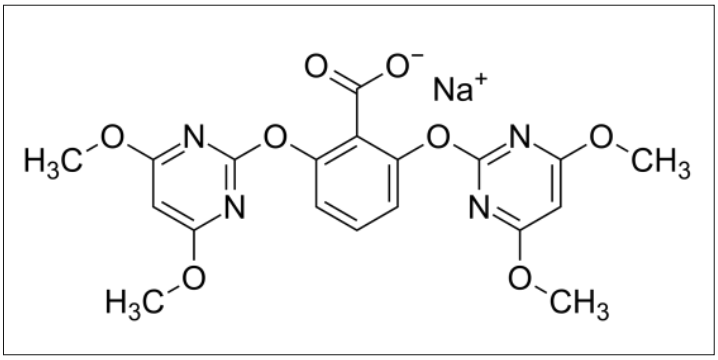

Figure 1.Structure of Bispyribac sodium

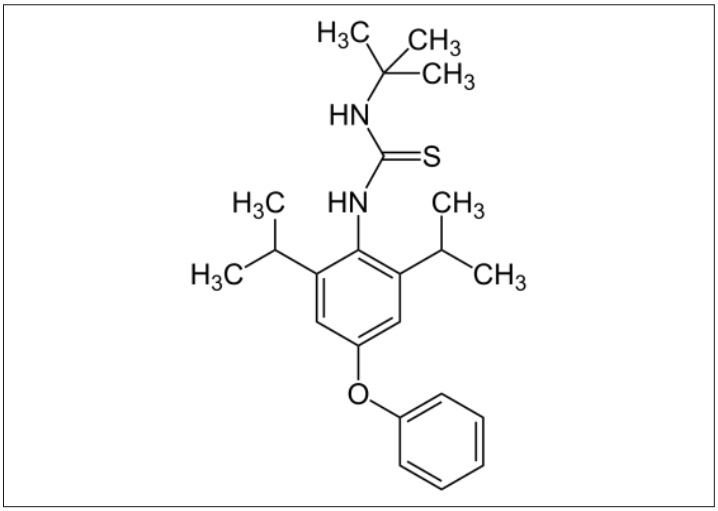

Figure 2. Structure of Diafenthiuron

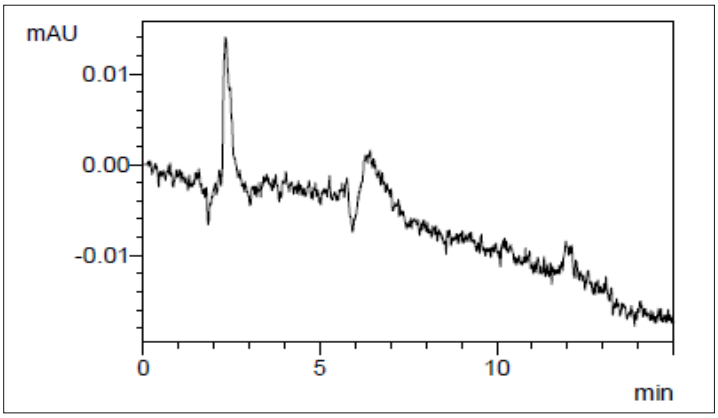

Figure 3.Chromatogram of blank (Bispyribac sodium)

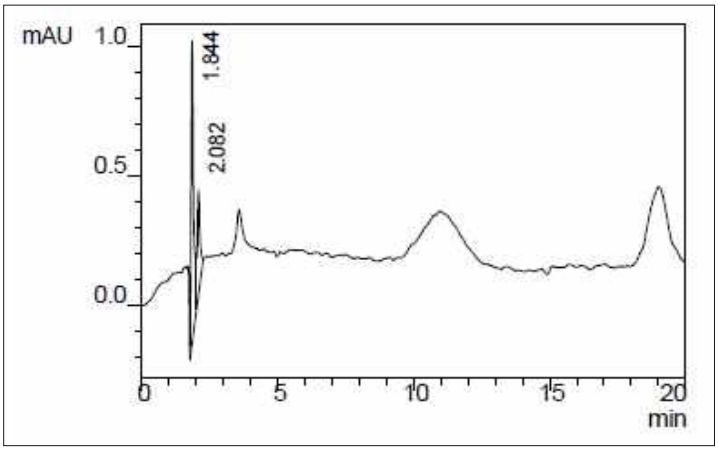

Figure 4.Chromatogram of blank (Diafenthiuron)

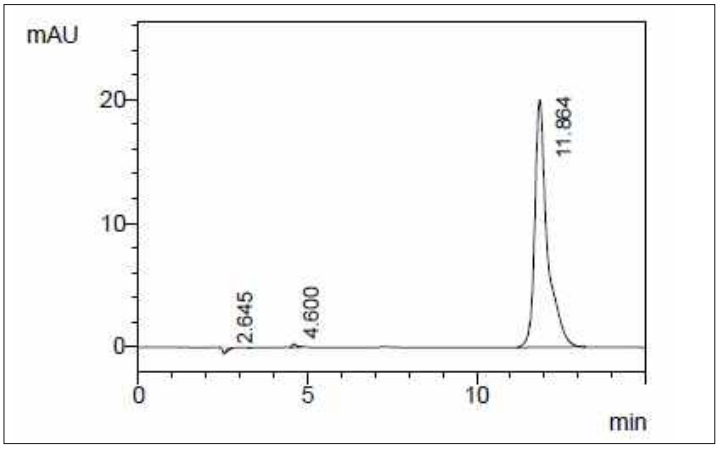

Figure 5.Chromatogram of standard preparation (Bispyribac sodium)

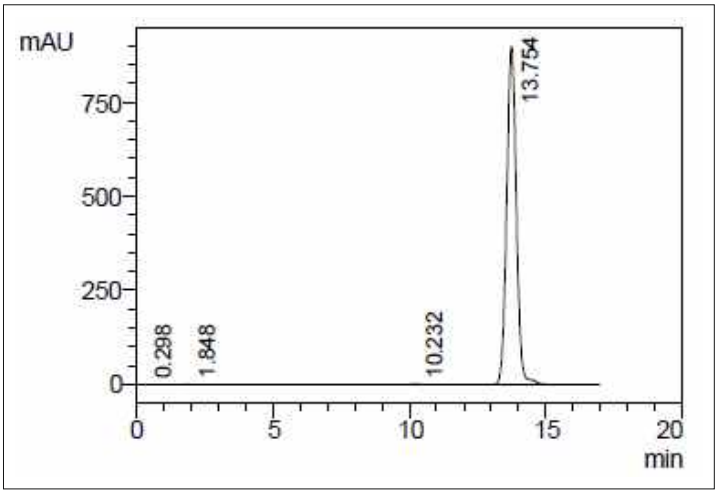

Figure 6.Chromatogram of standard preparation (Diafenthiuron)

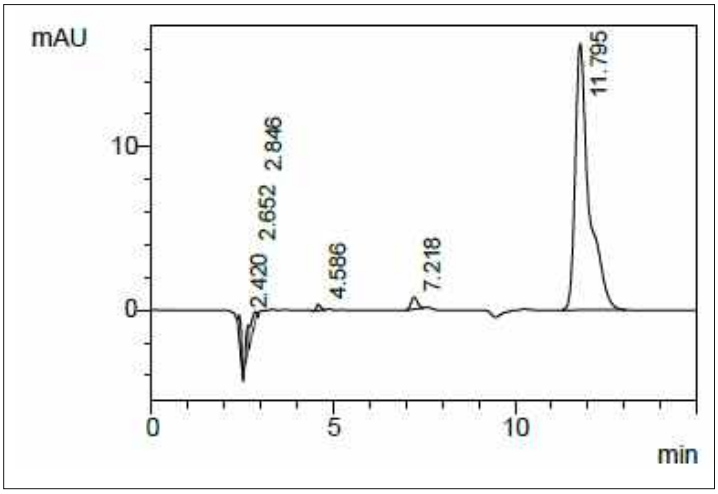

Figure 7.Chromatogram of sample preparation (Bispyribac sodium)

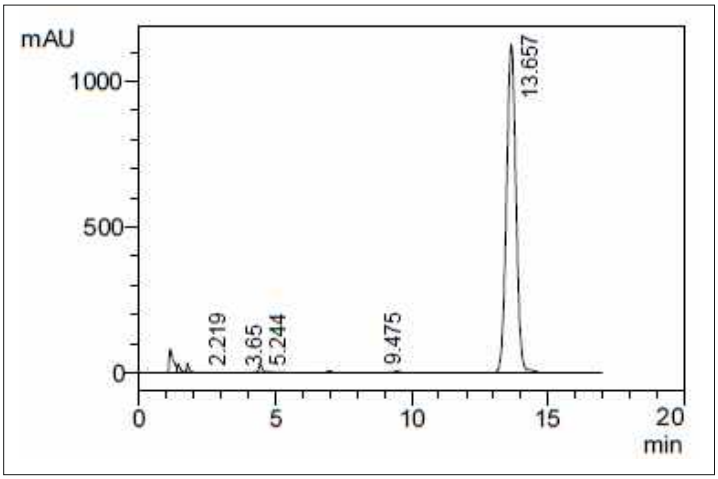

Figure 8.Chromatogram of sample preparation (Diafenthiuron)

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