ANALYTICAL METHOD VALIDATION REPORT FOR QUANTITATIVE ESTIMATION OF AMETOCTACTRADIN BY HPLC METHOD

N.Sarath Babu[1] & N.Venkatasubba Naidu[1]*
[1] DK Government Degree College for Women, Nellore-524 003, AP., India.
[1]*Department of Chemistry, S.V.University, Tirupati -517502, A.P., India.
E-mail:nvsn69@gmail.com

ABSTRACT

A simple, selective, precise and accurate high performance liquid chromatographic method for the analysis of Ametoctactradin in its formulations was developed and validated in the present study. The mobile phase consists of a mixture of methanol and water in the proportion 70: 30 (v/v) respectively This was found to give sharp peak of Ametoctactradin at a run time of 15 min. HPLC analysis of Ametoctactradin was carried out at a wave length of 294 nm with a flow rate of 1.0mL/min. The linear regression analysis data for the calibration curve showed a good linear relationship with a regression coefficient 0.999 in the concentration range of 50% to 150%. The linear regression equation was y=2984x+14.40. The developed method was employed with a high degree of precision and accuracy for the analysis of Ametoctactradin. The method was validated for accuracy, precision, robustness, ruggedness and specificity. The precision, accuracy, sensitivity, short retention time and composition of the mobile phase indicated that this method is useful for the quantification of Ametoctactradin.

Keywords: Ametoctactradin, HPLC Method, Development and Validation.

INTRODUCTION:

Fungicides are widely used in agriculture to protect crops, fruits and vegetables in the field and during the storage process. Therefore, the concentration of pesticide residues in many products, including fruits and vegetables, must be monitored. Their regulations have been developed [1] for food safety. Ametoctradin is chemically 5-ethyl-6 octyl[1, 2, 4]triazolo[1,5-a]pyrimidin-7- amine, empirical formula: $C_{15}H_{25}N_5$ and Molecular weight: 275.4 $g.ml^{-1}$. Ametoctradin is a fine crystalline solid. Ametoctradin solubility in water (water at 20 °C, pH 7): 0.15 mg/l. Ametoctradin is a mitochondrial

respiration inhibitor and belongs to a new class of chemistry, the pyrimidylamines. ZAMPRO^{TM} fungicide is a suspension concentrate formulation containing 300 g/l. It shows an excellent ecotoxicological profile. It is practically non toxic to birds, mammals, honeybees, earthworms and other soil macro-organisms, or to non-target soil micro-organisms and their ecosystem function. Ametoctradin is an active material of highly selective fungicides, such as Orvego, Resplend, Decabane, and Zampro, which provide an excellent tool for the effective control of diseases caused by oomycetes [2].

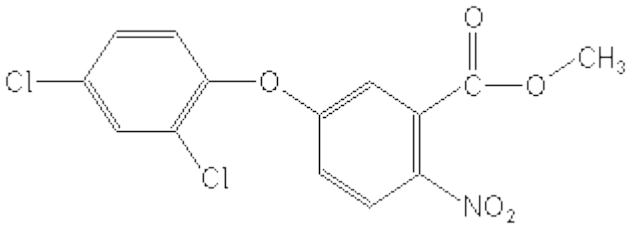


Figure: 1 Chemical structure of Ametoctradin

A survey of the literature revealed that different analytical techniques for the assay of Ametoctradin have been reported. Voltametric detection of the herbicide Ametoctradin at a bismuth film electrode in no deaerated solution1.Electroanalisis of Ametoctradin and metribuzen on lignin by Adsorption [3]. Determination of Ametoctradin in environmental samples and plant materials are based on the use of HPLC [4&5]. Electrochemical reduction of Ametoctradin [6]. The most accurate results in the analysis of samples of various cultures are provided by the application of mass spectrometric detection [7&8]. Development of analytical methods of spinosad in agricultural commodities by HPLC with UV detector and monitoring [9]**.**

In this study, an analytical method was developed and validated for the determination of Ametoctradin in its formulations. To the best of author knowledge, there is no one can report the determination of Ametoctradin in its formulations. An attempt has been made to develop and validate to ensure their accuracy, precision and other analytical method validation parameters as mentioned in various gradients for pesticide formulation, the proposed method is suitable for their analysis with virtually no interference of the usual additives presented in pesticide formulations.

INSTRUMENTS AND CHEMICALS

Instruments and Chemical used for the validation of Ametoctradin we used High performance liquid chromatography, with UV / PDA detector HPLC Analytical column of

Zorbax Extended - C18, 250mm x 4.6mm x 5µ and Analytical weighing balance - Mettler Toledo B204S, Millipore Nylon 0.2µm and Laboratory accessories. Ametoctradin working Standard, ZAMPRO^{TM} fungicide, Methanol- AR, Sodium Hydroxide – AR**,** Hydrochloric Acid – AR and Millipore Water [10]

ANALYTICAL METHOD

The quantitative determination is carried out by HPLC system equipped with UV/VIS detector.

Chromatographic conditions:

Column	**:**	Zorbax Extended - C18, 250mm x 4.6mm x 5µ
Mobile Phase	**:**	For isocratic system, prepare a mixture of Methanol and water in the proportion 70: 30 (v/v) respectively. Mix well. Filter through 0.2 µ Nylon membrane filter paper and degas prior to use.
Wavelength	**:**	294 nm
Flow Rate	:	1.0 ml / minute
Injection volume	:	20 µl
Run time	:	15 minutes
Blank solution	:	Use Methanol as blank
Diluent	:	Use Methanol as diluent

Preparation of Ametoctradin Standard Solution:

Weigh accurately about 50 mg of Ametoctradin working Standard and transfer to a 50 ml volumetric flask. Add 20 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix. (Dilution scheme: 50mg $\rightarrow$ 50.0 ml $\rightarrow$ 1 ml /10.0 ml)

Preparation of Test Solution:

Weigh accurately about 160 mg of sample and transfer to a 50 ml volumetric flask. Add 20 ml of diluent and sonicate to dissolve. Dilute to volume with diluent and mix. Transfer 1.0 ml of solution into a 10 ml of volumetric flask and dilute to volume with the diluent and mix. (Dilution scheme: 160mg $\rightarrow$ 50.0 ml $\rightarrow$ 1 ml /10.0 ml)

System Suitability Solution: Use Ametoctradin Standard working solution as system suitability solution.

Procedure:

Separately inject equal volumes of blank, five replicate injections of system suitability solution (Ametoctradin Standard working solution). Then inject two injections of test solution and record the chromatograms. Disregard any peak due to blank in the test solution. Calculate % RSD of five replicate injections of system suitability solution (Ametoctradin Standard working solution). Check tailing factor and theoretical plates of the peak in the chromatogram

obtained with 5th injection of system suitability solution (Ametoctradin Standard working solution). The limits are as below,

1. Theoretical plates should be not less than 2000.
2. Tailing factor should be less than 2.0.

VALIDATION PARAMETERS [10]

Specificity / Selectivity:

Selectivity was performed by injecting the diluent blank solution, excipient blend, system suitability solution, test solution. Acceptance criteria: The Ametoctradin peak should be well resolved from any other peak and from each other. The diluent blank solution, excipient blend solution should not show any peak at the retention time of the Ametoctradin. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.

Table - 2: System suitability - Selectivity

Sr. No.	Area of Ametoctradin
1	2761.76
2	2744.74
3	2768.11
4	2744.67
5	2751.24
Mean	**2754.10**
Standard Deviation (±)	**10.49**
(%) Relative Standard Deviation	**0.38**

All the injections were processed at the wavelength provided in the method. There was no interference observed from diluent blank solution, excipient blend solution with Ametoctradin peak.

FORCED DEGRADATION

The forced degradation studies are performed to establish the stability indicating nature of the assay method and to observe any degraded compounds. Ametoctradin WS and Sample (ZAMPRO™ Fungicide) are subjected to stress with 5N HCl, 5N NaOH, Thermal degradation and UV degradation. All the above solutions are chromatographed and recorded the chromatograms. The following stress conditions are followed for degradation

Table – 3: System suitability – Forced Degradation

Sr. No.	**Area of Ametoctradin**
1	2855.28
2	2863.80
3	2859.42
4	2888.71
5	2837.66
Mean	**2860.97**
Standard Deviation (±)	**18.41**
(%) Relative Standard Deviation	**0.64**

Table – 4: Conditions – Forced Degradation

Sample stress condition	**Description of stress condition**
Acid degradation	5N HCl heated at about 60°C for 10 min on a water bath.
Alkali degradation	5N NaOH heated at about 60°C for 10 min on a water bath.
Thermal degradation	105ºC for 12 hours
UV degradation	expose to UV-radiation for 7 days

Table-4 : % of degradation by applying different conditions

Acid Stress	**% Degradation**
Standard	0.010
Sample	0.011
Alkali Stress	**% Degradation**
Standard	0.414
Sample	0.612

Thermal Stress	**% Degradation**
Standard	0.778
Sample	0.884
UV Stress	**% Degradation**
Standard	0.006
Sample	0.037

Acceptance Criteria: The degradation peaks should be well separated from each other. The peak purity for Ametoctradin peak should pass. There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Ametoctradin peak is passing. Hence, the method is very precise, selective and specific to the estimation of Assay of Ametoctradin in ZAMPRO^{TM} FUNGICIDE by HPLC and the same method is stability indicating, as the degraded products are well separated from Ametoctradin and as well from each adjacent peaks.

Linearity and Range for sample:

For the linearity study five sample solutions of Ametoctradin were prepared from the range starting from 50% to 150% of the theoretical concentration of assay preparation. The system suitability solution and the linearity solutions were injected as per the protocol. The linearity graph of concentration against peak response was plotted and the correlation coefficient was determined. Acceptance criteria: Correlation coefficient should be greater than or equal to 0.999. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table-5 for system suitability results).

Table 5: System suitability - Linearity of sample

Sr. No.	**Area of Ametoctradin**
1	2812.89
2	2813.82
3	2842.39
4	2869.96
5	2838.85
Mean	**2835.58**

Standard Deviation (±)	**23.60**
(%) Relative Standard Deviation	**0.83**

The average peak area of Ametoctradin peak at each concentration level was determined and the linearity graph was plotted against the sample concentration in percentage. The results of linearity study are as given in Table 6.

Table 6: Results of linearity of standard

Linearity Level	Sample Concentration(in %)	Sample Concentration(inppm)	Peak Area	Correlation Coefficient
Level – 1	50	50	1496.94	**0.999**
Level – 2	75	75	2162.52	
Level – 3	100	100	2865.11	
Level – 4	125	125	3649.94	
Level – 5	150	150	4371.81	

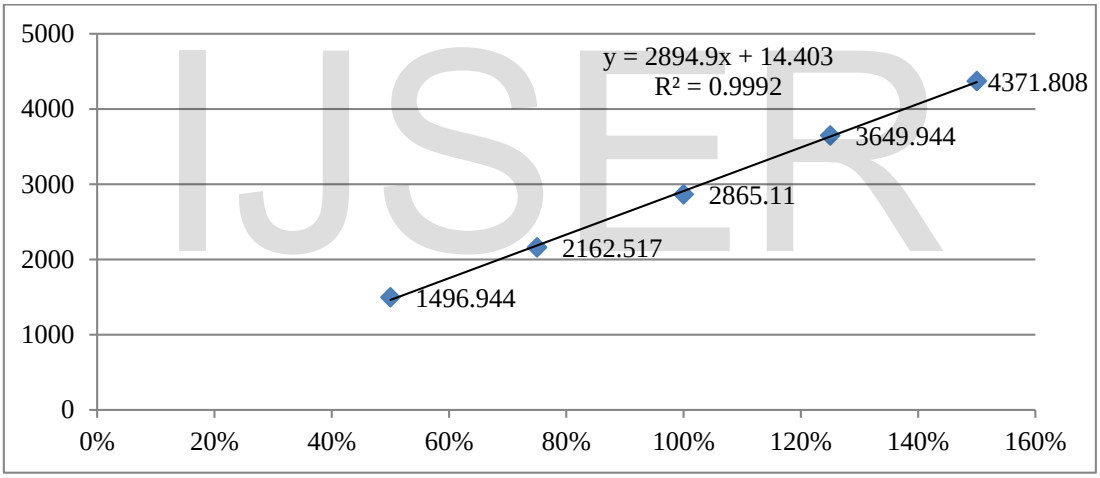
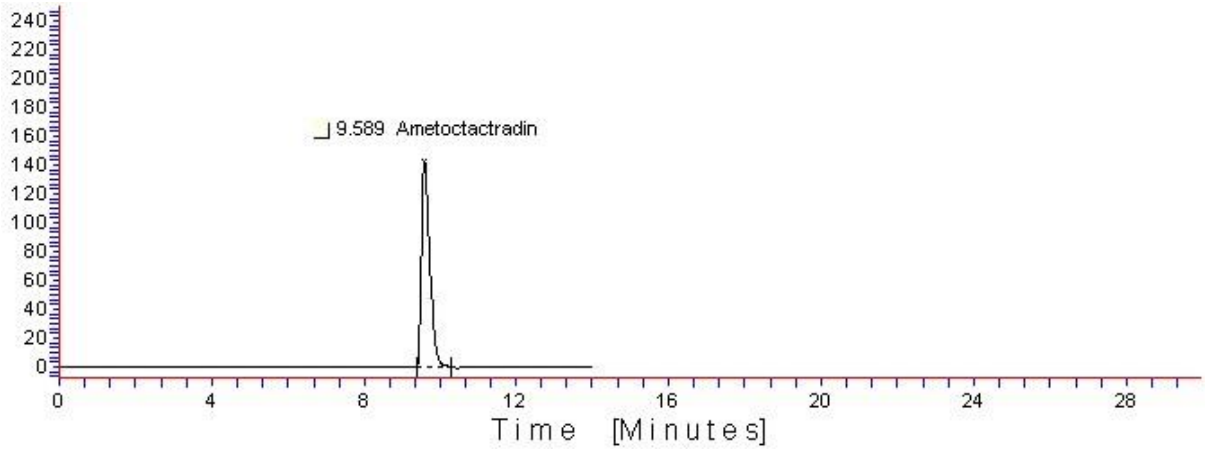


Figure 2: Linearity graph of Ametoctradin sample



Result-A Table						
Peak No	**Retn.Time**	**Area**	**Height**	**Area %**	**Height %**	**Width@50%**
1	9.589	2162.517	143.329	100	100	0.25
Total		**2162.517**	**143.329**	**100**	**100**	

Figure 3: Chromatogram of Ametoctradin sample

PRECISION:

System Precision:

Procedure:

The system precision was performed by injecting 10 replicate injections of system suitability solution and the chromatograms are reviewed for the system suitability criteria. Acceptance criteria: % RSD of peak areas of ten replicate injections of system suitability solution should not be more than 2.0% and system suitability criteria should pass as per analytical method. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method.

Table 7: System precision

Sr. No.	**Area of**
1	2875.97
2	2893.58
3	2886.85
4	2889.69
5	2867.68
6	2866.20
7	2875.15
8	2842.66
9	2856.64
10	2884.29
Mean	**2873.87**
Standard Deviation (±)	**15.95**
(%) Relative Standard Deviation	**0.55**

Method Precision:

Procedure:

Six test solutions of Ametoctradin in ZAMPRO^{TM} FUNGICIDE and were prepared as per the analytical method. The % RSD of % assay of six test solutions was calculated. Acceptance criteria: % RSD of the results of six test solutions should not be more than 2.0%. The system suitability criterion was found to meet the pre-established acceptance criteria as per the analytical method. The results of assay obtained from six test solutions preparations are presented in Table - 9

Table - 8: System suitability - Method precision

Analyst – 1 HPLC No.: EH/R&D/HPLC-024

Sr. No.	Area of Ametoctradin
1	2758.58
2	2738.25
3	2719.22
4	2776.75
5	2749.58
Mean	**2748.47**
Standard Deviation (±)	**21.58**
(%) Relative Standard Deviation	**0.79**

Table - 9: Results of method precision

Test Solution	% Assay of Ametoctradin
1	101.39
2	101.73
3	103.01
4	100.91
5	100.73
6	100.53
Mean	**101.38**
Standard Deviation (±)	**0.91**
(%) Relative Standard Deviation	**0.90**

Intermediate Precision:

Procedure:

Six test solutions of ZAMPRO^{TM} fungicide was prepared as per the analytical method on different day. These test solutions were analyzed by a different analyst using different HPLC column of same make but having different serial number and different HPLC system.

The % RSD of % assay results of twelve test solutions (six samples from method precision and six samples from intermediate precision) was calculated. Acceptance criteria: % RSD of the results of twelve test solutions (six of method precision and six of intermediate precision) should not be more than 2.0%. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical method. (Refer to Table -10 for system suitability results). The results of assay obtained from six test solutions are presented in Table - 11. % RSD of assay results from method precision and intermediate precision (12 results) are presented in Table - 12.

Table - 10: System suitability - Intermediate precision

Analyst – 2 HPLC No.: EH/R&D/HPLC-023

Sr. No.	**Area of Ametoctradin**
1	2717.67
2	2704.27
3	2723.11
4	2726.38
5	2716.66
Mean	**2717.62**
Standard Deviation (±)	**8.46**
(%) Relative Standard Deviation	**0.31**

Table - 11: Results of Intermediate precision

Test Solution	**% Assay of Ametoctradin**
1	98.38
2	98.54
3	100.23
4	99.55
5	99.83
6	99.22
Mean	**99.29**
Standard Deviation (±)	**0.73**
(%) Relative Standard Deviation	**0.73**

Table - 12: Results of twelve test solutions of Ametoctradin in ZAMPRO™ fungicide (six of method precision & six of intermediate precision)

Analysis performed during method precision study By Analyst 1 on system 1 and on column 1 on day 1	
Same column	**% Assay of Ametoctradin**
1	101.39
2	101.73
3	103.01
4	100.91
5	100.73
6	100.53
Analysis performed during intermediate precision study By Analyst 2 on system 2 and on column 2 on day 2	
Column sr. no.	**015337030136 02**
Test Solution	**% Assay of Ametoctradin**
7	98.38
8	98.54
9	100.23
10	99.55
11	99.83
12	99.22
Mean of twelve samples	**100.34**
Standard Deviation (±)	**1.35**
(%) Relative Standard Deviation	**1.34**

The analysis was carried out on six test solutions of the same lot of the drug product by two different analysts using two different equipments within the same laboratory using two different columns of the same make but having different serial numbers on two different days. The % RSD of the twelve assay results (six of method precision and six from intermediate precision) is found to be less than 2.0%. Thus, the method is found to be rugged and precise.

Robustness:

Procedure

Prepare two test solutions of the same lot (as used in 7.0.a and 7.0.b) of Ametoctradin in ZAMPRO^{TM} fungicide as per analytical method. Inject this solution along with diluent blank solution and system suitability solution along different chromatographic conditions as shown below:

Change in flow rate (± 0.2 ml/minute)

Change in wavelength (± 2 nm)

Change in composition of mobile phase (± 20ml)

Change in Flow Rate (± 0.2 mL/minute):

(Normal Experimental Condition: 1.0ml/minute)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method. (Refer to Table - 15 for system suitability results).

Table - 15: System suitability - Robustness with change in flow rate

Sr. No.	**Area of Ametoctradin**	
	0.8mL/minute	**1.2 mL/minute**
1	2866.32	2815.58
2	2881.16	2805.84
Mean	**2873.74**	**2810.71**
Standard Deviation (±)	**10.49**	**6.88**
(%) Relative Standard Deviation	**0.37**	**0.24**

The assay results obtained with different flow rate conditions are as given in Table 16.

Table - 16: Results for change in flow rate

Flow rate →	**0.8mL/minute**	**1.2 mL/minute**
Sample	**% Assay**	
Test solution	99.91	100.03
Average assay result from Method precision	101.38	101.38
Mean	**100.65**	**100.71**
Standard Deviation (±)	**1.04**	**0.95**
(%) Relative Standard Deviation	**1.03**	**0.95**

Change in Wavelength (± 2 nm):

(Normal Experimental Condition: 294nm)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method. (Refer to Table - 17 for system suitability results).

Table - 17: System suitability - Robustness with change in wavelength

Sr. No.	**Area of Ametoctradin**	
	292 nm	**296 nm**
1	2765.01	2753.38
2	2767.01	2745.47
Mean	**2766.01**	**2749.43**
Standard Deviation (±)	**1.41**	**5.59**
(%) Relative Standard Deviation	**0.05**	**0.20**

The assay results obtained with different wavelength conditions are as given in Table - 18.

Table - 18: Results for change in wavelength

Wavelength →	**292 nm**	**296 nm**
Sample	**% Assay**	
Test solution	99.68	100.32
Average assay result from Method	101.38	101.38
Mean	**100.53**	**100.85**
Standard Deviation (±)	**1.20**	**0.75**
(%) Relative Standard Deviation	**1.20**	**0.74**

Change in composition of Mobile Phase (± 20ml):

(Normal Experimental Condition: Methanol : water = 700ml:300ml)

The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method (Refer to Table - 19 for system suitability results).

Table - 19: System suitability - Robustness with change in composition of mobile phase

Sr. No.	**Area of Ametoctradin**

	720ml:280ml	**680ml:320ml**
1	2841.46	2845.78
2	2865.09	2827.50
Mean	**2853.27**	**2836.64**
Standard Deviation (±)	**16.71**	**12.93**
(%) Relative Standard Deviation	**0.59**	**0.46**

The assay results obtained with change in composition of mobile phase are as given in Table - 20.

Table - 20: Results for change in composition of mobile phase

Composition of Methanol & water	**720ml:280ml**	**680ml:320m**
Sample	**% Assay**	
Test solution	100.03	100.11
Average assay result from Method precision	101.38	101.38
Mean	**100.71**	**100.75**
Standard Deviation (±)	**0.95**	**0.90**
(%) Relative Standard Deviation	**0.95**	**0.89**

The analysis of the same lot of ZAMPRO^{TM} fungicide was carried out at different conditions of column lot, flow rate, wavelength, and change in composition of mobile phase. The system suitability was found to meet the pre-established criteria at all the conditions and the % RSD between results obtained with changed condition and average result of Method precision is not more than 2.0%. The analytical Method meets the pre-established acceptance criteria for robustness study as per protocol. Thus, the Method is robust.

Stability of Analytical Solution:

Procedure:

System suitability solution and test solution of ZAMPRO^{TM} fungicide were prepared on 0th, 12th, 24th, 36th and 48th hour of experiment and stored these solutions at room temperature for every time interval up to 48 hrs and analyzed these solutions on 48 hrs with freshly prepared test solution. The system suitability solution was prepared freshly at the time of analysis. The assay of ZAMPRO^{TM} fungicide in the sample was calculated. Acceptance criteria: The analyte is considered stable if there is no significant change in % assay. The assay results obtained during solution stability experiment are as given in Table- 20.

Table - 20: Results for solution stability

% Assay results calculated against the freshly prepared system suitability standard	
Sample	**% Assay of Ametoctradin**
0^{th} hr	99.34
12^{th} hr	98.78
24 hr	99.73
36 hr	98.51
48 hr	98.32
Mean	**98.94**
Standard Deviation (±)	**0.59**
(%) Relative Standard Deviation	**0.59**

RESULTS AND DISCUSSION:

System selectivity:

All the injections were processed at the wavelength provided in the Method. There was no interference observed from diluents blank solution, excipients blend solution with Ametoctactradin peak. The system suitability criteria were found to meet the pre-established acceptance criteria as per the analytical Method. Hence this Method is selective

Forced degradation:

There is no interference between the peaks obtained for the chromatograms of degradation preparations. The degradation peaks under forced degradation are well separated from each other. The peak purity for Ametoctactradin peak is passing. Hence, the Method is very precise, selective and specific to the estimation of Assay of Ametoctactradin in test solution of ZAMPRO^{TM} Fungicide as a Ametoctactradin 99% by HPLC and the same method is stability indicating, as the degraded products are well separated from Ametoctactradin and as well from each adjacent peak.

Linearity:

Linearity graph of the average area at each level against the concentration in methanol and water in the proportion 70: 30 (v/v) is plotted and is found to be a straight line graph. The correlation coefficient is found to be more than 0.999. Hence it is concluded that the method is found to be linear in the range of 50% to 150% of the working concentration.

Precision:

The analysis was carried out on six test solutions of the Ametoctactradin in ZAMPRO^{TM} Fungicide and by two different analysts using two different equipments within the same laboratory using two different columns of the same make but having different serial numbers on two different days. The % RSD of the twelve assay results which six of method

precision and six from intermediate precision is found to be less than 2.0%. Thus, the method is found to be rugged and precise.

System precision=%RSD=0.55

Method precision=%RSD=0.90

Intermediate precision=%RSD=0.73

Robustness:

The analysis of the Ametoctactradin in ZAMPRO^{TM} Fungicide 99% was carried out at different conditions of column lot, flow rate, wavelength, and change in composition of mobile phase., The % RSD between results obtained with changed condition and average result of Method precision is not more than 2.0%.The analytical Method meets the reestablished acceptance criteria for robustness study. Thus, the Method is robust.

Stability of Analytical Solution:

The %RSD between assay results obtained for freshly prepared test solution and the stored test solutions is less than 2.0%there is no significant change in assay level observed up to 48 hours for test solution at room temperature. The system suitability was found to meet the pre-established criteria and it can be concluded that the solution is stable up to 48 hours at room temperature

SUMMARY AND CONCLUSION:

The above summary and the validation data summarized in this paper show the analytical method of assay of Ametoctactradin in ZAMPRO^{TM} Fungicide 99% by HPLC is found to be suitable, selective, specific, precise, linear, accurate and robust. The analytical solution is found to be stable up to 48 hours at room temperature. Hence, it is concluded that the analytical method is validated and can be used for routine analysis and for stability study.

ACKNOWLEDGEMENTS

The authors are thanks to Analog labs Hyderabad, India for providing Ametoctactradin pesticide and Department of Chemistry, Sri Venkateswara University, Tirupati, India for providing laboratory facilities.

REFERENCES

1. Mingfeng Hu, Xingang Liu, Fengshou Dong, Jun Xu, Shasha Li, Hanqing Xu et al. Determination of ametoctradin residue in fruits and vegetables by modified quick, easy, cheap, effective, rugged, and safe method using ultra-performance liquid chromatography/tandem mass spectrometry. Food Chemistry. 2015; 175:395-400.

2. Gold, R., Scherer, M., Rether, J., Speakman, J., Nave,B., Levy, T., Storer, R., and Marris, D., in Proc. BCPC Congress, Glasgow, UK, 2009, p. 1.
3. Uclés.S, Belmonte.N, Mezcua.M… - … Science and Health. - Taylor & Francis, 2014.
4. Do, J.-A., Kwon, J.-E., Lee, E.-M., Kim, M.-R., Kuk,J.-H., Cho, Y.-J., Kang, I.-H., Kim, H.-S., Kwon, K., and Oh, J.-H., Korean J. Food Sci. Technol., 2013, vol. 45, no. 3, p. 285.
5. Yukhnik, A.V. and Prismotrov, Yu.A., in Zdorov'e i okruzhayushchaya sreda: sbornik nauchnykh trudov (Health and Environment: A Collection of Scientific Papers), Minsk, 2012, no. 20, p. 308
6. Zaouak.A, Matoussi.F, Dachraoui.M - Journal of Environmental, Taylor & Francis, 2013.
7. Hu, M., Liu, X., Dong, F., Xu, J., Li, S., Xu, H., and Zheng, Y., Food Chem., 2015, vol. 175, p. 395.
8. Martinez, G., Morales, A., Maestro, A., Cermenj, S., Oliva, J., and Barba, A., J. AOAC Int., 2015, vol. 98, no.6, p. 1745
9. Kwon CH, Lee YD, Im MH.. J. Agr. Food Chem. 59: 10826-10830 (2011)
10. ICH Guideline number: Q2A & Q2B of CPMP / ICH / 281 / 95.