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Analytical method development for combination pesticides of picloram and fluroxypyr by reverse phase High Performance Liquid Chromatography (HPLC)

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ABSTRACT

The herbicide is the part of the pesticide, these herbicide are normally used to control the weeds in the cultivation period. The quality of the herbicide has to be effective and the degradation should be fast. After the usage, the soil fertility has to be maintained without any environmental chain changes. There are much herbicides are available to control weeds or unwanted plants in the cultivation processes. Many of the herbicides are used to control the grass or very small plants, which has a very week in the structure. The Picloram herbicide used to control especially the plant which has strong stem and branches. The usage is unique and this herbicide has to be used carefully to maintain the required cultivated plants during the plantation or cultivation period. There are few herbicides are available to control woody plants. The speciality of the picloram herbicide will not have any activity on the grass. Fluroxypyr is another molecule being used as herbicide in the cultivation industry. The chemical nature of this molecule is similar to that of the picloram molecule. The Fluroxypyr is consisting of the pyridine molecule basically. Fluroxypyr has fluorine, chlorine, amine and an ether arrangement in its structure. This Fluroxypyr is used to control woody plant which has a broad leaf. This molecule also has the electronegative structural annulments in it has more penetration power through roots of the plants. Mostly this Fluroxypyr is being used to control weeds in the place where the cultivations are normally carried out. These Picloram + Fluroxypyr molecules were separated through a mobile phase consisting of the mixture of acetonitrile and water (fortified with 0.5% of formic acid) in the ratio of 40:60 v/v. The separation was achieved through the Qualisil BDS C8 (250 x 4, 5µ) column with the flow rate as 1.5 ml/min with the detection at 254 nm. These method parameters were loaded in the Shimadzu HPLC (model: LC-2030). The LC solution Shimadzu software was used for all the calculations in this analytical method validation analysis. The results of the study showed that the proposed RP-HPLC method is simple, rapid, precise and accurate, which is useful for the identification and quantifications of these molecules interims of validation parameters viz., separation, system suitability, System Precision and linearity in a simple HPLC analysis.

Keywords— *Picloram + Fluroxypyr, HPLC analysis, Validated method, SANCO 3030/99 Rev.4, ICH Guideline*

1. INTRODUCTION

The herbicide is the part of the pesticide, these herbicide are normally used to control the weeds in the cultivation period. The quality of the herbicide has to be effective and the degradation should be fast. After the usage, the soil fertility has to be maintained without any environmental chain changes. There are many herbicides are available to control weeds or unwanted plants in the cultivation processes. Many of the herbicides are used to control the grass or very small plants, which has a very week in the structure. The Picloram herbicide used to control especially the plant which has strong stem and branches. The usage is unique and this herbicide has to be used carefully to maintain the required cultivated plants during the plantation or cultivation period. There are few herbicides are available to control woody plants. The speciality of the picloram herbicide will not have any activity on the grass.

Such a very special molecule picloram has to be understood better chemically. This picloram molecule was a derivative of the picolinic acid. In the picolinic acid, all the carbons of the pyridine were substituted by three chlorine and one amine. Naturally, the chlorine atoms are electronegative in nature and the substituted amine basic in nature. The structural arrangement of the Picloram has high penetrating power anywhere on the earth crest. So the Picloram molecule will be easily travelling in the water and will penetrate through roots of the woody plants. The results of this will disturb the photosynthesis of the plants. This is the processes of killing all the woody plants by using Picloram herbicide.

The combination herbicide Picloram and Fluroxypyr, one of the best herbicide and the analytical method has to be developed to estimate effectively and accurately with lost cost and time. Hence an effective HPLC method was developed to estimate these molecules at a single injection.

2.MATERIALS AND METHOD

2.1 Reagents and chemicals used

All the analytical grade solvents and water were used in this analytical method development. All the class A glass were used in this research analytical method development.

2.2 Instrument

A calibrated chromatography HPLC instrument was used to develop this analytical method development for Fluroxypyr 80g/L and Picloram 80g/L. The instrument parameters were given as:

Name of the instrument	:	High Performance Liquid Chromatography (HPLC)
Calibration Method	:	External Standard Method
Make	:	Shimadzu
Model	:	LC 2030
Detector	:	UV-Visible
Wavelength (λ)	:	254 nm
Column Temperature	:	40ºC
Column	:	Qualisil BDS C8 (250 x 4.6 mm, 5µ)
Mobile Phase	:	Acetonitrile: Water (0.5% Formic acid); ratio (40:60) v/v
Flow rate	:	1.5 ml/min
Injection volume	:	20 µl
Retention time (Approximately)	:	Picloram – 3.5 minutes; Fluroxypyr – 5.4 minutes
Total Run Time	:	10 minutes

2.3 Preparation of mobile phase

A volume of 40% Acetonitrile and 60% of 0.5% Formic acid were mixed well, sonicated and used for analysis.

3. ANALYTICAL METHOD VALIDATION

3.1 Specificity

3.1.1 Preparation of standard stock solutions: An amount of 10.06 mg of Picloram reference standard with purity 99.5% and 10.11 mg of Fluroxypyr reference standard with purity 99.0% was weighed accurately into a clean and dry 10 mL volumetric flask separately and dissolved in mobile phase and made up to the mark with the mobile phase. This was equivalent to 1000.97 mg/L and 1000.89 mg/L. From this, each 2.5ml solution was added in 25 ml volumetric flask and diluted with mobile phase. This solution was equivalent to 100 mg/L and used for determination of specificity.

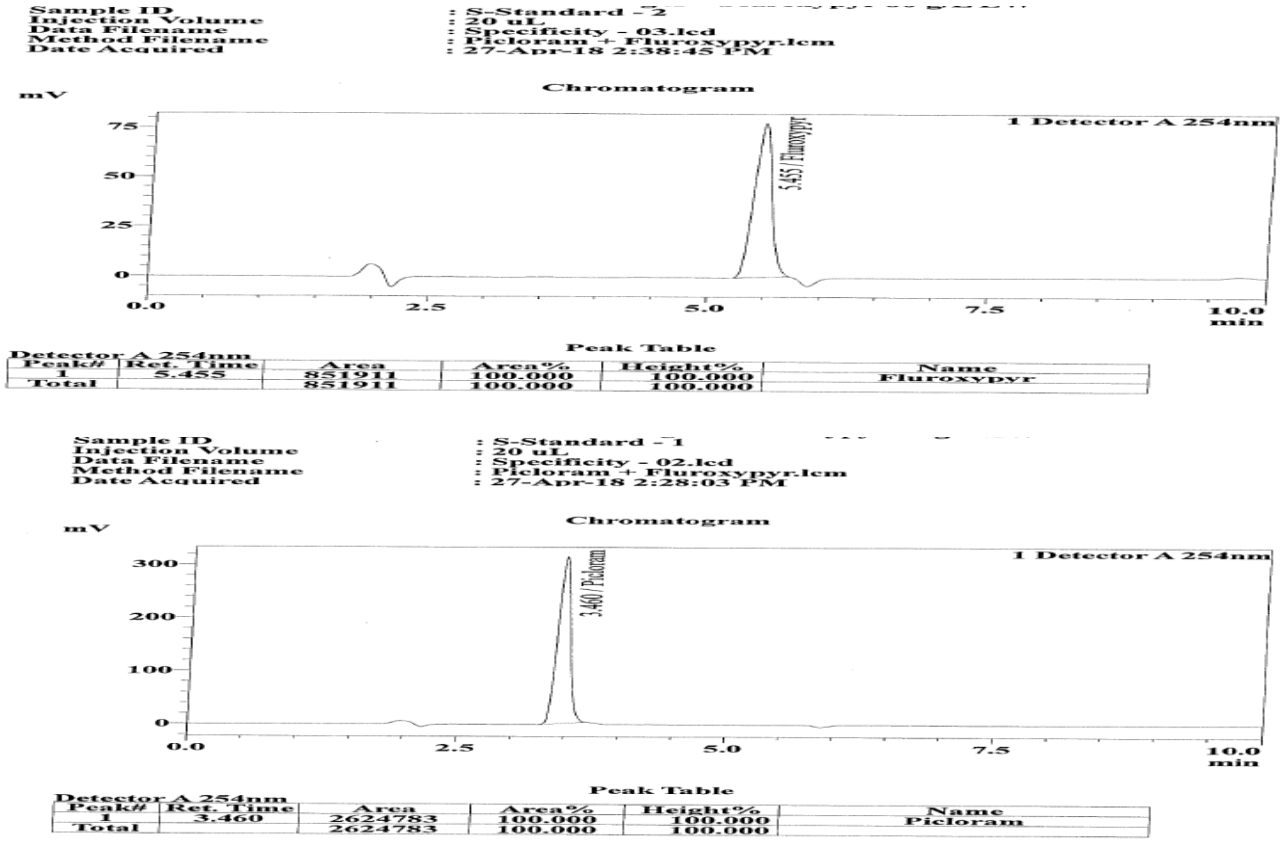


Fig. 1: A typical chromatogram for specificity (Picloram and Fluroxypyr)

3.1.2 Preparation of sample solution: An amount of 10.0 mg of the test substance was weighed accurately into a clean and dry 100 mL volumetric flask and dissolved in mobile phase and made up to the mark with the mobile phase. This was equivalent to 100 mg/L. This prepared solution was used for the determination of Specificity.

The specificity of HPLC method for Picloram and Fluroxypyr was determined by injecting the Standard and Sample solutions along with blank (mobile phase) and observed that there was no interference found with the main peak of interest. Hence, this method was considered to be specific for the analysis of Picloram and Fluroxypyr.

3.2 Linearity

3.2.1 Preparation of standard stock solution and working standard: An amount of 10.0 mg of the standard was weighed into a 100 ml standard flask and this concentration (100 mg/L) was used to prepare further dilutions to get the 0.2, 1, 10, 30, 60 and 90 mg/L separately. The dilution details are presented in table 1.

Table 1: Dilutions (Picloram 80G/L + Fluroxypyr 80G/L reference standard)

Standard Code	Stock Dose (mg/L)	Dilution Volume (ml)	Final Volume (ml)	Final Concentration (mg/L)
Stock	1000	2.5	25	100
1	100	0.02	10	0.2
2	100	0.1	10	1
3	100	1.0	10	10
4	100	3.0	10	30
5	100	6.0	10	60
6	100	9.0	10	90

The prepared standard solutions were injected by an autosampler into the HPLC system and a linear curve was plotted for the concentration of standard versus observed peak area and the correlation coefficient was determined respectively. The results are presented in table 2 and table 3.

Table 2: Linearity of Picloram reference standard

Std. Code	Concentration (mg/L)	Replication	Ref. Std. Area	Mean Std. Area
Std-1	0.2	R1	4652	4506
		R2	4359	
Std-2	1	R1	11072	10944
		R2	10815	
Std-3	10	R1	270993	271869
		R2	272744	
Std-4	30	R1	792542	793616
		R2	794689	
Std-5	60	R1	1574032	1574601
		R2	1575170	
Std-6	90	R1	2405407	2404849
		R2	2404291	
			Intercept	-6269.8779
			Slope	26663.1918
			Correlation Coefficient	0.9999

Table 3: Linearity of Fluroxypyr reference standard

Std. Code	Concentration (mg/L)	Replication	Ref. Std. Area	Mean Std. Area
Std-1	0.2	R1	2328	2429
		R2	2529	
Std-2	1	R1	7220	7421
		R2	7622	
Std-3	10	R1	94551	93554
		R2	92556	
Std-4	30	R1	283590	284273
		R2	284955	
Std-5	60	R1	601067	601385
		R2	601703	
Std-6	90	R1	865128	864853
		R2	864577	
			Intercept	-1155.6588
			Slope	9732.4631
			Correlation Coefficient	0.9996

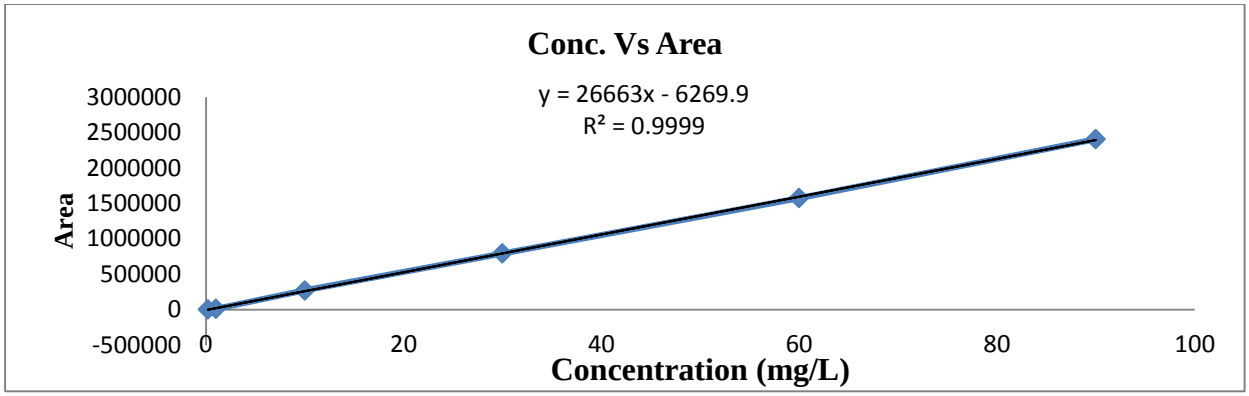


Fig. 2: Linearity curve for Picloram

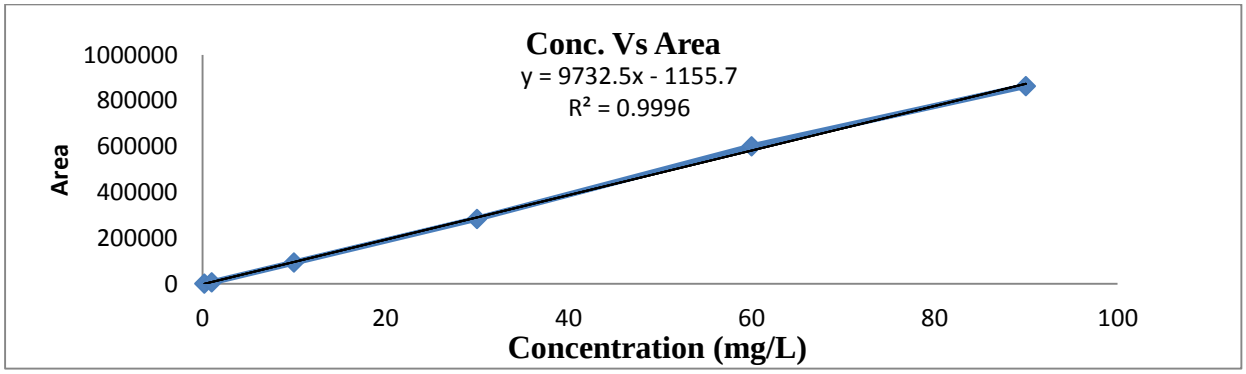


Fig. 3: Linearity curve for Fluroxypyr 80G/L

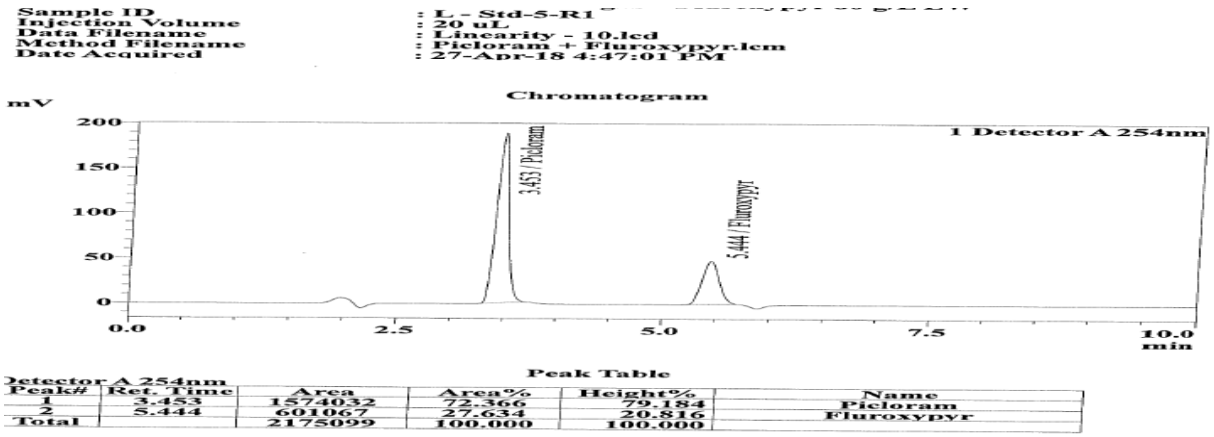


Fig. 4: A typical chromatogram for linearity (Picloram and Fluroxypyr)

4.PRECISION

4.1 Preparation of standard solution

The Linearity standard solution 5 mg/L was prepared and used for the precision determination.

4.2 Preparation of sample solution

An amount of 5.95, 5.80, 5.81, 5.90 and 6.0mg of Picloram + Fluroxypyr Technical was weighed in clean and dry 1000 ml volumetric flask separately, dissolved the contents with mobile phase and made upto the mark with the mobile phase. This solutions are equivalent to 59.5, 58.0, 58.1, 59.0 and 60.0mg/L. The prepared solutions were injected into HPLC and % RSD was calculated and the results are presented in table 4.

Table 4: Precision (Picloram)

Sample ID	Std. Conc. (mg/L)	Std./Sample Area	Average Std. Area	Sample Conc. (mg/L)	Purity (P) %	A.I. Content (%)
Std -R1	10	274478	275366.5		99.5	-
P1		796441		355.0		8.11
P2		798308		354.0		8.15
P3		800462		356.0		8.12
P4		798358		358.0		8.06
P5		797927		355.0		8.12
Std - R2		276255				-
					Mean	8.11
					SD	0.034
					% RSD	0.415

Table 5: Precision (Fluroxypyr 80G/L)

Sample ID	Std. Conc. (mg/L)	Std./Sample Area	Average Std. Area	Sample Conc. (mg/L)	Purity (P) %	A.I. Content (%)
Std -R1	10	92275	93943.0		99.0	-
P1		271721		355.0		8.07
P2		271512		354.0		8.08
P3		271701		356.0		8.04
P4		271777		358.0		8.00
P5		271659		355.0		8.06
Std - R2		95611				-
					MEAN	8.05
					SD	0.032
					% RSD	0.396

Formula:

$$\text{A. I. Content (\%)} = \frac{\text{Sample Area x Std. Conc. (mg/L)}}{\text{Average Std. Area x Sample Conc. (mg/L)}} \times \text{Purity (P) \%}$$

The % RSD is within limit according to the modified Horwitz equation (Acceptable Limit <1.3% RSD for 100% active analyte as per SANCO/3029/99 Rev.4)

5.ACCURACY (% RECOVERY)

The recovery processes and the recovery determination was validated with two fortification level of processes.

5.1 Preparation of standard solution

The standard solution prepared for linearity (10 mg/L) was used as a standard in per cent recovery determination.

5.2 Preparation of fortification level 1 (58 mg/L)

An amount of 5.83 mg of Picloram reference standard with purity 99.5% and 5.86 mg of Fluroxypyr reference standard with purity 99.0% was weighed accurately in to a clean and dry 100 mL volumetric flask contains 50 ml of distilled water, sonicated and made up to the mark with the distilled water. This solution was equivalent to 58 mg/L.

5.3 Preparation of fortification level 2 (45 mg/L)

An amount of 4.52 mg of Picloram reference standard with purity 99.5% and 4.55 mg of Fluroxypyr reference standard with purity 99 % was weighed accurately in to a clean and dry 100 mL volumetric flask contains 50 ml of distilled water, sonicated and made up to the mark with the distilled water. This solution was equivalent to 45 mg/L. These prepared solutions were used for % recovery determination.

The above preparations were analyzed under HPLC and checked for recovery (%). The results are presented in following table 6 and table 7.

Table 6: Accuracy (Level-1 and 2 Recovery %) of Picloram

Fortification Level	Std. Conc. (mg/L)	Std./Sample area	Mean Std. Area	Recovery Conc. (mg/L)	Fortified Conc. (mg/L)	Recovery (%)	Avg. Recovery (%)
Std-R1	10	275967	275768.0	-	58.0	-	-
T1R1		1588310		57.60		99.30	99.24
T1R2		1586663		57.54		99.20	
T1R3		1587519		57.57		99.25	
T1R4		1587212		57.56		99.23	
T1R5		1586676		57.54		99.20	
T2R1		1250076		45.33	45.0	100.73	100.71
T2R2		1249953		45.33		100.73	
T2R3		1249429		45.31		100.68	
T2R4		1249472		45.31		100.69	
T2R5		1249817		45.32		100.71	
Std - R2		275569		-		-	

Table 7: Accuracy (Level-1 and 2 RECOVERY %) OF FLUROXYPYR 80G/L

Fortification Level	Std. Conc. (mg/L)	Std. / Sample area	Mean Std. Area	Recovery Conc. (mg/L)	Fortified Conc. (mg/L)	Recovery (%)	Avg. Recovery (%)
Std-R1	10	94044	95567.0	-	58.0	-	-
T1R1		541874		56.701		97.76	97.95
T1R2		563300		58.943		101.63	
T1R3		536233		56.111		96.74	
T1R4		535834		56.069		96.67	
T1R5		537494		56.243		96.97	

T2R1		429921		44.986		99.97	
T2R2		431692		45.172		100.38	
T2R3		428802		44.869	45.0	99.71	100.13
T2R4		428612		44.849		99.67	
T2R5		433984		45.411		100.91	
Std - R2		97090		-		-	

Example Calculation: **Recovery (Picloram) T2R5**

$$\text{Recovery Conc. (mg/L)} = \frac{\text{Std. Conc.}\,(mg/L) \times \text{Sample area}}{\text{Mean Std. Area}} = \frac{5 \times 1249817}{5 \times 1249817} = 45.32$$

$$\text{Recovery (\%)} = \frac{\text{Recovery Conc. (mg/L)}}{\text{Fortified Conc (mg/L)}} \times 100 = \frac{45.32}{45.0} \times 100 = 100.71\%$$

6.LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTIFICATION (LOQ)

From the Linearity Standard Solution concentration of 10 mg/L was used in these LOD & LOQ determinations. From this solution, 1 mg/L solution was prepared and further diluted to get the 0.02 & 0.1 mg/L concentration solutions were prepared. The dilution details were given in table 8, and the results are presented in following table 9 and table 10.

Table 8: Dilutions (LOD and LOQ)

Stock concentration (mg/L)	Dilution Volume (ml)	Final Volume (ml)	Final Concentration (mg/L)
1.0	2.0	10	0.2
0.2	1.0	10	0.02

Formula:

LOD = Average + (3 x Standard Deviation)
LOQ = Average + (10 x Standard Deviation)

Table 9: Limit of Detection (LOD) and Limit of Quantification (LOQ) of picloram

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	A. I. Content (mg/L)
STD-1		276233		-
R1		463		0.017
R2	10	475	275631.0	0.017
R3		534		0.019
STD-2		275029		-
			Mean	0.0178
			SD	0.00138
			LOD	0.02

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	A. I. Content (mg/L)
STD-1		276233		-
R1		4815		0.175
R2	10	4667	275631.0	0.169
R3		4436		0.161
STD-2		275029		-
			Mean	0.168
			SD	0.00693
			LOQ	0.24

Table 10: Limit of Detection (LOD) and Limit of Quantification (LOQ) of Fluroxypyr

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	A. I. Content (mg/L)
STD-1		94297		-
R1		249		0.0260
R2	10	237	95951.0	0.0247
R3		272		0.0283
STD-2		97605		-
			Mean	0.0263
			SD	0.00185
			LOD	0.03

Sample ID	Std. Conc. (mg/L)	Std./ Sample Area	Average Std. Area	A. I. Content (mg/L)
STD-1		94297		-
R1		2426		0.253
R2	10	2400	95951.0	0.250
R3		2477		0.258
STD-2		97605		-
			Mean	0.254
			SD	0.00408
			LOQ	0.29

Example calculation: (LOD and LOQ)
Formula:

LOD = Average + (3 x Standard Deviation).
LOQ = Average + (10 x Standard Deviation)

Example calculation: (LOD and LOQ)
Limit of Detection (Fluroxypyr) R1

$$\text{A. I. Content (mg/L)} = \frac{\text{Std. Conc. (mg/L)} \times \text{Sample Area}}{\text{Average Std. Area}}$$

$$\text{A. I. Content (mg/L)} = \frac{10 \times 249}{95951} = 0.0260 \text{ mg/L}$$

LOD = Mean Value + (3 × SD)
LOD = 0.0263 + (3 × 0.00185) = 0.03

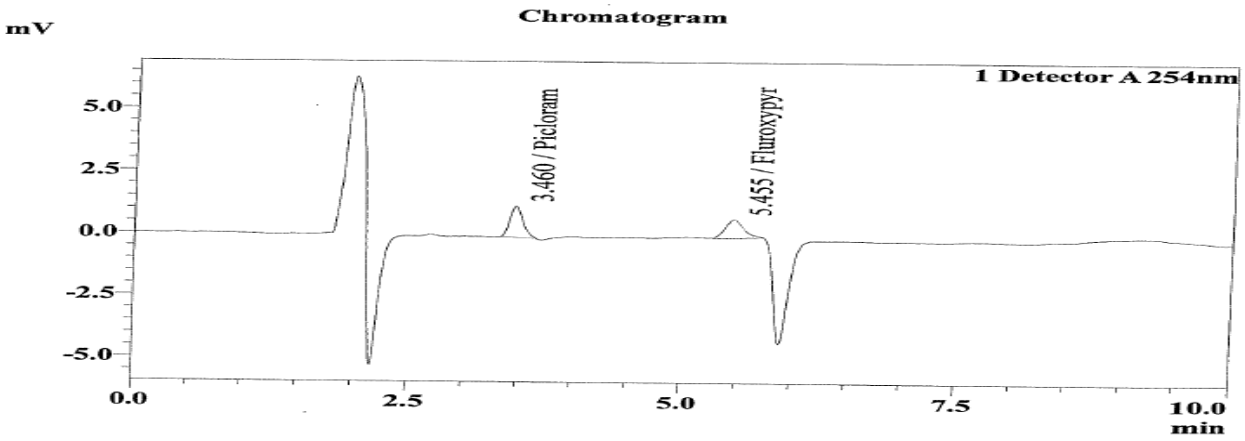
Limit of Quantification (Fluroxypyr) R1

$$\text{A. I. Content (mg/L)} = \frac{\text{Std. Conc. (mg/L)} \times \text{Sample Area}}{\text{Average Std. Area}}$$

$$\text{A. I. Content (mg/L)} = \frac{10 \times 2426}{95951} = 0.253 \text{ mg/L}$$

LOQ = Mean Value + (10 × SD)
LOQ = 0.254 + (10 × 0.00408) = 0.29

Sample ID	**: L - Std-2-R1**
Injection Volume	**: 20 uL**
Data Filename	**: LOD - LOQ -08.lcd**
Method Filename	**: Picloram + Fluroxypyr.lcm**
Date Acquired	**: 27-Apr-18 3:42:51 PM**



Peak Table

Detector A 254nm

Peak#	Ret. Time	Area	Area%	Height%	Name
1	3.460	4667	66.039	65.256	Picloram
2	5.455	2400	33.961	34.744	Fluroxypyr
Total		7067	100.000	100.000	

Fig. 5: A typical chromatogram for LOD and LOQ (Picloram and Fluroxypyr)

7. ACTIVE CONTENT ANALYSIS OF PICLORAM 80G/L + FLUROXYPYR 80G/L

7.1 Preparation of standard solution

An amount of 5 mg of the standard was dissolved in 100 ml of mobile phase and diluted to get 10 mg/L was used as a standard in concentration analysis.

7.2 Preparation of sample solutions

The combination sample solution was prepared 200 mg/mL concentrations and diluted to further about 1.6 times dilution appropriately and injected into HPLC with the validation set of instrumental parameters.

$$\text{Picloram 80g/L} + \text{Fluroxypyr 80g/L (mg/L)} = \frac{\text{A x B x DF}}{C}$$

Where,
A: Concentration of standard (ppm)
B: Area of the sample solution
C: Area of standard solution
DF: Dilution Factor

8. CONCLUSION

8.1 Specificity

The blank, standard and the sample peaks did not interfere with each other, hence the specificity was achieved as per the guideline SANCO 3030/99 Rev.4 requirement.

8.2 Linearity

The Linearity correlation coefficient is achieved NLT 0.99 as per (SANCO 3030/99 Rev.4).

8.3 System precision

The system precision is achieved as the % RDS as 0.145 for 5 replicates observed as 0.1% for Picloram + Fluroxypyr , hence the minimum requirement of the (SANCO 3030/99 Rev.4 was NMT 15% RSD was achieved).

8.4 System recovery

The system recovery 97.95 to 100.71 % were achieved for Picloram + Fluroxypyr, hence the minimum requirement of the (SANCO 3030/99 Rev.4).

8.5 Details of the laboratory work were carried out

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