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Method Development and Validation for the Determination of Clodinafop-propargyl (EC/WP Formulation) by HPLC-UV-Visible Technique

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Abstract

Wheat (Triticum aestivum L.) is a major staple food of Pakistan and contributes about 1.6% of the GDP. In Pakistan, the area under wheat crop is approximately 9.16 million hectares with an overall production of 27.46 million tons annually. It meets the main dietary requirements and feeds about 220.8 million people in the country. The infestation of different weeds is a serious problem for wheat crop production in Pakistan. Generally, it causes a yield loss of about 40% to 60%.

Background: Herbicide samples are tested for the determination of their active ingredients (a.i), but various methods give different results. Thus, the main purpose of the current study was to develop and validate a Clodinafop-propargyl determination method by using the HPLC-UV-Visible technique.

Methods: The HPLC-UV-Visible technique used for the quantification of Clodinafop-propargyl contents in the herbicide sample was validated in the Pesticide Quality Control Laboratory (ISO/IEC: 17025), Bahawalpur. This method validation process includes: Use of Blank, Use of Reference Standard, Specificity, repeatability, reproducibility, Detection Limit (LOD), Quantification Limit (LOQ), Linearity, Measurement Uncertainty, Ruggedness and recovery.

The detection and quantification limits of the current method were 2.4 and 8.0ppm of Clodinafop-propargyl, respectively. The repeatability, however, was 0.1547 %, and the reproducibility (calculated T- was 0.653) was less than T-tabulated (i.e., 2.262). The Linear curve was found for the concentration ranging from 200 to 1000 ppm Clodinafop-propargyl, showing R^2 of 1. As for recovery is concerned, it was 100.030% for Clodinafop-propargyl in the herbicide sample. The Clodinafop-propargyl concentration was detected with complete recovery without any interference from other materials in the sample. The Z-scores for all PT samples were within an acceptable range. The coefficient of correlation (1%) exhibited a strong correlation between actual Clodinafop-propargyl contents and observed contents.

Conclusions: Based on the above findings, it is assumed that the performance of the method under study was excellent. Therefore, this method can be effectively employed for Clodinafop-propargyl estimation in herbicides.

Keywords: Clodinafop-propargyl, Development, Validation, Method, HPLC

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Introduction

Wheat (Triticum aestivum) is the most extensively cultivated crop, being the food grain crop of the world, and it play crucial role in worldwide food security as it provides food for billions of people as well as half of the dietary protein (Meena et al., 2017). Various biotic and abiotic factors directly impact wheat growth. Among those, the most dangerous one is weed infestation. The yield loss due to weed infestation is very high, even 17-30% annually (Rao and Chauhan, 2015). Different herbicides are used to control weeds in wheat (Chachar et al., 2009). The chemical method is a major one as it is a rapid, much more effective, and time-saving method for weed control in wheat crops (Mehmeti et al., 2018). The critical justification for a validated and modified HPLC method for the determination of Clodinafop-propargyl herbicide is scientifically essential in order to have a quality herbicide for proper weed control.

Clodinafop-propargyl is a post-emergence weedicide for weed control in cereal crops such as wheat. But for the best quality of Clodinafop-propargyl herbicide, its method of determination needs to be validated. In modern chemistry, high-performance liquid chromatography is a potent analytical tool. It is very good at recognizing, quantifying, and separating. elements in samples that have been dissolved in a liquid. HPLC is widely used in various product analyses and is highly valued for its accuracy in both quantitative and qualitative evaluations, greatly advancing analytical chemistry (Rao et al., 2015). In HPLC, a porous column is filled with a sample solution (the stationary phase). Next, a high-pressure liquid (mobile phase) is injected through the column. Partitioning between the stationary and mobile phases causes components in the sample to migrate across the column at varying speeds. Elution occurs at different times as a result, enabling separation.

The accuracy of HPLC results from subtle component behaviours during partitioning, providing a reliable technique for examining a variety of samples in many domains (Rajan et al., 2015). A compound with a lower affinity for the stationary phase moves more quickly and covers a greater distance in high-performance liquid chromatography, whereas a molecule with a higher affinity moves more slowly and covers a shorter distance. Effective sample component separation and analysis are made possible by this differential migration (Kumar et al., 2015).

According to the standard (ISO/IEC: 17025), for any analytical method, its validation is inevitable to guarantee that this analytical method is capable of fulfilling the suitable criteria. The main aim of the current study was to validate the HPLC method for Clodinafop-propargyl determination in herbicide samples by changing temperature (i.e., 30 ^{0}C and 35 ^{0}C) and by changing the Flow rate from 1.0 ml to 1.2 ml.

Materials and Methods

Development and validation of the method were carried out through the assessment of several analytical figures of merit according to the International Conference on Harmonization, including Specificity, repeatability, reproducibility, Detection Limit (LOD), Quantification Limit (LOQ), Linearity, Uncertainty Measurements, Ruggedness, and recovery (Guideline, 2007; Sahoo et al., 2018). The current study regarding method validation was conducted at the Pesticide Quality Control Laboratory, Bahawalpur, Pakistan.

Accuracy

By definition, the accuracy is ''closeness of the results to the actual value". For the accuracy determination of the method, the repeatability data of two different analysts were used. According to the Collaborative International Pesticides Analytical Council (1999), the best validated technique is considered one having an accuracy value > 85%. The accuracy was calculated by using the formula of Desta and Amare (2017) & Sinshaw et al. (2019).

Accuracy = 100 – error.

Error (%) = Observed value – True value X 100

True value

Precision

The results of repeatability and reproducibility were used to calculate precision, which is defined as "agreement among a set of replicated measurements without knowledge of true value." For repeatability, analyst-1 prepared ten samples with the same concentration of Clodinafop-propargyl and measured its contents; for reproducibility, analyst-2 prepared samples with the same concentration of Clodinafop-propargyl and ran them on HPLC for ten repetitions (Barnawal et al., 2016)

Limit of Detection and Limit of Quantification

The limit of detection (LOD) is the lowest quantity of any material that can be detected and clearly differentiable from zero, but not certainly quantified (González et al., 2018; McDowall, 2005). The quantification limit (LOQ) is the lowest concentration of any material that is measurable quantitatively with a satisfactory level based on precision and accuracy (González et al., 2018; González and Herrador, 2007; Markley et al., 1998).

Measurement of uncertainty

For uncertainty measurement, the Eurachem Guide was employed. The uncertainty in the measurement results could be because of various factors, e.g., person, analysis protocol, environment, as well as different equipment, chemical and glass apparatus. Combined uncertainty is the combined effect of all the factors. The uncertainty budget comprises all different uncertainties because of above stated factor (Cortez, 1995; Örne mark, 2004). Uncertainty is measured at 68% confidence level. According to ISO/IEC: 17025, the testing labs are urged to report their system-related uncertainties with a defined level of confidence that is expressed as the "expanded uncertainty" (Aslam et al., 2021; Nazir et al., 2020; van der Veen and Cox, 2021).

Combined uncertainty = $\sqrt{(U_{(x1)})^{2} + (U_{(x2)})^{2} + (U_{(x3)})^{2} + (U_{(x4)})^{2}}$.

Expanded uncertainty = Combined uncertainty x confidence level

Equipment/Apparatus Required:

HPLC-UV-Visible, Analytical Balance, Ultrasonic Bath

Reagents/Medias Required

Acetonitrile (HPLC Grade)

Trifloroaceticacid

De-ionized Water

Clodinafop propargyl std. Of known purity

Method

Identification

Retention time of standard and sample should be comparable + 3%.

Preparation of Standard Solution:

Weighed accurately 50.0 mg ± 0.5 mg standard into 25ml vol. flask, also added 15ml of acetonitrile, and then sonicated for 10 minutes. Allowed to cool to ambient temperature and then made volume with acetonitrile.

Preparation of Sample Solution:

In the case of SC formulation

Weigh an appropriate quantity of sample containing 50.0mg ±1.0 mg (w mg) of active ingredient into a 25 ml volumetric flask, and add 15 ml of mobile phase. Allow to cool to ambient temperature and then make volume with acetonitrile.

HPLC Conditions for Analysis:

Column	C18, 150 x 4.6mm, having 5µm particles or equivalent
Detector	UV VWR 305 nm
Column Temperature	40^0C
Mobil Phase/Eluent	Acetonitrile 60 ml + 40 ml Dist. Water (0.5 % TFA)
Flow Rate	1 mL /min
Quantity of Inject	10µl

Procedure:

Under the chromatographic operating conditions, when the baseline of the instrument became stable, the standard and sample solutions were injected, and three readings of the standard and two readings of the samples were taken independently. RSD of three consecutive standard areas should be ≤1.3% as per the Horwitz principle. Calculated the %age of the product as below:

Calculation:

%Age purity (w/w) = Peak Area of the sample X Weight of Standard X Purity of Standard

Average Peak Area of Standard X Weight of Sample

Cautions/Safety Requirements:

Always inject the filtered sample into the HPLC.

Wash the HPLC system with a suitable solvent for a minimum of 30 minutes before shutting down the system.

Always add acid to water and avoid putting water into acid.

Criteria for Validation of Method Performance:

Standard methods need verification, while non standard laboratory developed method needs validation in order to ensure that the laboratory is capable of performing analysis. Validation or Verification of any analytical method is the demonstration that a laboratory is proficient in replicating a non-standard or standard method with an acceptable level of performance. Validation under conditions of use is confirmed by meeting the system fitness, requirements, accuracy and precision. Method performance is achieved by employing performance features such as:

Use of blank, Use of reference standard, Specificity, Repeatability, Reproducibility, detection limit, Quantification Limit, Linearity, Measurement of uncertainty, Ruggedness & Robustness, Recovery/Accuracy.

Results

Use of Blank

Before Each Injection Auto zero being done by the system automatically.

Reference standards

Table-1: Standards Information

Standard identification	**Purity**
Clodinafop Propargyl	95%

Specificity and Selectivity

The selectivity of the analytical protocol refers to the degree to which a certain technique of analysis is stable for determining the availability of specific analytical parameters in any mixture without any interferences due to other parameters in the mixture. For the determination of the selectivity of the current method, CRM of Clodinafop Propargyl (98.2 % purity) was run on HPLC. After three injections, a sample of the mixture, including active ingredients, was analyzed. The Peak shape of the active ingredient was clear, and the retention time of the active ingredient (A.I) in the

sample was similar to that of CRM. The Selectivity data is presented in Table No.2. The content was recognized without significant interference of other nutrients in the mixture according to the formulation, therefore passes.

Table 2: Specificity and Selectivity

S No.	Sample ID	Area of Standard	Area of sample	Result (%w/w)
1	R1	2307	2295	14.95
2	R2	2307	2298	14.98
3	R3	2307	2297	14.97

Repeatability

The nearness of the agreement among independent results was obtained with the same protocol on identical material of testing, under the same conditions (same analyst, same equipment, same lab and after a short time interval). The measurement of repeatability was the Relative Standard Deviation, qualified with the terminology: `repeatability' as repeatability RSD. The data of 10 replications (Table-3) predicted that the method under study is repeatable with the relative standard deviations of ±0.1547 %, including instrument precision and sample homogeneity for clodinafop propargyl, which is fairly less than 15%; hence, the parameter is categorized as pass.

Table-3: Repeatability Measurement

S/N	Observations	CLODINAFOP PROPARGYL(%)
1.	Reading 1	14.9
2.	Reading 2	14.9
3.	Reading 3	14.9
4.	Reading 4	14.9
5.	Reading 5	14.9
6.	Reading 6	14.9
7.	Reading 7	14.9
8.	Reading 8	14.9
9.	Reading 9	14.9
10.	Reading 10	14.9
	Average	14.91
	SD	0.0231
	RSD	0.1547

Reproducibility

The data (Table-4) explain the nearness of the agreement between independent Clodinafop Propargyl results gained with a similar method on identical testing material but under dissimilar conditions (different analyst, different environmental conditions and after different time intervals). The measure of reproducibility was the standard deviation represented with the terminology "reproducibility" as reproducibility SD, examined with the application of the t-test in this study. As per the t-test, the technique (method) is capable of delivering reproducible results, whereas duplicating analyses with an average standard deviation of ±0.022% for Clodinafop Propargyl.

Table-4: Reproducibility Measurement

S. No.	Observation	CLODINAFOP PROPARGYL		
		Analyst-I (%)	Analyst-II (%)	SD (%)
1	Reading 1	14.90	14.90	0.0014
2	Reading 2	14.91	14.90	0.0109
3	Reading 3	14.94	14.94	0.0018
4	Reading 4	14.94	14.93	0.0078
5	Reading 5	14.92	14.91	0.0046
6	Reading 6	14.93	14.92	0.0077
7	Reading 7	14.94	14.94	0.0018
8	Reading 8	14.89	14.88	0.0109
9	Reading 9	14.88	14.88	0.0014
10	Reading 10	14.90	14.90	0.0046

11	**Average**	14.91	14.91	0.0365
12	**SD**	0.0212	0.0231	0.00528

t-Test

Table-5: t = (V1-V2) / (SQRT ((SD1²/10)+(SD2²/10))

t-Test	**CLODINAFOP PROPARGYL**	
	14.91	14.91
SD	0.021174	0.023062
(SD)2/10	0.000045	0.000053
(SQRT((S.D)2/10)+(SD)2/10)	0.009900481	
V1-V2	0.006468118	
T	0.653	

t (tabulated-t)= 2.262 at 95% level of confidence

As calculated t-value 0.653 is far less than tabulated-t value (i.e., 2.262), thus, reproducibility is considered to be successful; henceforward, the parameter is classified as pass.

Method Limit of Detection (LOD)

The method detection limit (LOD) by definition is the lowest concentration of any material that can be measured as well as reported with 99% level of confidence that the analyte concentration is larger than, and is determined by the analysis of a sample in a given material containing the typical analyte. The LOD in the current study was obtained as 2.4 ppm for clodinafop propargyl. The study is conducted by analysis of a trial sample following the procedure of the Eurachem laboratory guide. The data of 10 samples was employed for the determination of LOD (Table-6).

Method Limit of Quantification (LOQ)

The LOQ is the lowest quantity of any analyte which can be determined with satisfactory performance. In practice, LOQ is calculated by most conventions to be the analyte concentration corresponding to the obtained standard deviation at lower levels multiplied by a factor of 10. The LOQ in this study is obtained as 8.0 ppm for clodinafop propargyl. The LOQ limit is achieved after analysis of the trial sample by following the procedure of the Eurachem laboratory guide (Table-6).

Table-6 Evaluation of Limits of Detection (LOD) and Limits of Quantification (LOQ)

S/N	Observations	CLODINAFOP PROPARGYL (ppm)
1	Reading1	100
2	Reading2	101
3	Reading3	100
4	Reading4	100
5	Reading5	100
6	Reading6	99
7	Reading7	100
8	Reading8	100
9	Reading9	99
10	Reading10	100
	Avg	99.9
	SD (So)	0.56
	Śo= so*SQR (2)	0.80
	LOD=3* Śo	2.4
	LOQ=10* Śo	8.0

Linearity

Linearity is the condition where the dependent variables have a direct relationship with the independent variables; therefore, they can be calculated as a linear function of the independent variables. In Figure-1, the relationship between concentration and the standard Area of the sample is drawn up to the maximum method capacity and is still linear. Linearity is also characterized by 'good behaviour and predictability', with R2 =1.0, which is >0.995 value as required by the criteria, hence, this parameter is marked as pass.

Table-7 linearity of clodinafop-propargyl

Readings	Concentration (ppm)	Area
1	0	0
2	100	460
3	200	920
4	300	1379
5	400	1839
6	500	2298
7	600	2757
8	700	3215
9	800	3672
10	900	4136

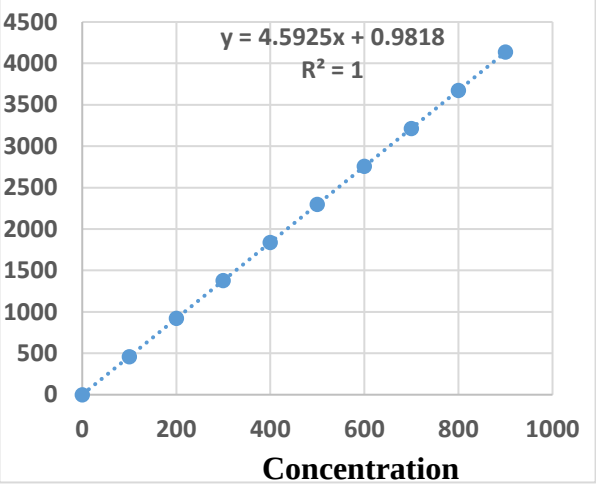

Figure-1: Linear Curve of clodinafop propargyl

Uncertainty Measurement (Budget)

Annexure (A)

The state of being doubtful or the doubt in measurements is stated at K=2 (95% probability) and is estimated as ±0.14% for clodinafop propargyl that can be regarded as best for the said method at PQCL, Bahawalpur, as per the decision rules in conformity assessment, particularly for ISO/IEC 17025:2017 standard.

Uncertainty Measurement (Budget)

Replications	Analyst 1	Analyst 2	Mean		Difference	Normalized Difference	Uncertainty (U_{Prec})
	(A1)	(A2)	(A1+	A2)	(A1-A2)	(Difference/	SD of Normalized
1	14.90	14.90	29.804	17	14.90	0.0001994977	0.0001	**0.0002582**
2	14.91	14.90	29.808	65	14.90	0.0001541366	0.0010	
3	14.94	14.94	29.873	47	14.94	0.0002484808	-0.0002	
4	14.94	14.93	29.868	99	14.93	0.0001096205	0.0007	
5	14.92	14.91	29.825	62	14.91	0.0006471717	0.0004	
6	14.93	14.92	29.856	03	14.93	0.0001095729	0.0007	
7	14.94	14.94	29.873	47	14.94	0.0002484808	-0.0002	
8	14.89	14.88	29.77		14.88	0.0001539354	0.0010	
9	14.88	14.88	29.75		14.88	0.00	0.0001	
10	14.90	14.90	29.799	7	14.90	0.0006465555	0.0004	
			Mean		**14.912**	**SD**	0.0004473 8	

			{ C }				

Ruggedness (Robustness)

The 'ruggedness' ('robustness') of any analysis method is "a measure of its ability to remain unaffected by very small, but deliberate changes in the protocol parameters. Ruggedness provide an indicator of the reliability of the method during normal use". The effect on clodinafop propargyl results variations (Table-09) are within the defined tolerance limit (±5%).

Table-9. Ruggedness and Robustness Study

Test item mean value on normal conditions			15	
Lower Control Limit			14.1	
Upper Control Limit			14.9	
Sr. No.	**CLODINAFOP PROPARGYL**			
	Result (%) at Temp. 30^{0}C	Result (%) at Temp. 35^{0}C	Result Difference (%)	Tolerance range for verification (±2.5)
1	14.93	14.94	-0.01	Pass
2	14.89	14.88	0.01	Pass
3	14.91	14.91	0.00	Pass
Sr. No.	**CLODINAFOP PROPARGYL**			
	Result (%) at F.R 1.0ML	Result (%) at F.R 1.2ML	Result Difference (%)	**Tolerance range for verification (±2.5)**
1	14.93	14.91	0.02	Pass
2	14.89	14.94	-0.05	Pass
3	14.91	14.93	-0.02	Pass

Recovery

Recovery was calculated from linearity of working standards by the following formula:-

% Recovery/Accuracy = Found Concentration *10 Claim Concentration

Found Concentration = Peak Area-Intercept Slope

Table 10: recovery measurement

Std. Conc. (ppm)	Std Area	Found Conc.	%Recovery
0	0	0.00	0.00
100	460	99.91	99.91
200	920	200.17	100.08
300	1379	300.08	100.03
400	1839	400.30	100.07
500	2298	500.26	100.05
600	2757	600.21	100.04
700	3215	699.95	99.99
800	3672	799.47	99.93
900	4136	900.52	100.06
		Mean Recovery	100.030
		SD	0.062

Discussion

Method validation is inevitable to cater to the requirements of ISO/IEC 17025. In order to achieve precise and accurate results from any analytical method, it is mandatory to validate the method in question in the local environment. The method must be capable of fulfilling the suitable criteria to generate accurate results. The current method of Clodinafop-propargyl herbicide determination through High Performance Liquid chromatograph was validated in the Pesticide Quality Control Laboratory, Bahawalpur. All the required protocols were used during validation. Repeatability is a basic parameter which tells us whether the method is capable of producing replicated results or not. The repeatability value of the method was 0.1547, which indicates that the method is capable of producing replicated results. Similarly, reproducibility is unavoidable to check whether the method can reproduce the results under dissimilar conditions or not. For the T-test applied, the

Sr. No.	Validation Parameter	Criteria	Result	Remarks
1	Blank	-	-	Auto zero
2	CRM	Available	Complies	Pass
3	Specificity	-	-	Pass
4	Repeatability	RSD < 15%	0.1547	Pass
5	Reproducibility	t value < 2.262	T-value=0.653	Pass
6	LOD	Should be calculated	2.4 ppm	Pass
7	LOQ	Should be calculated	8.0ppm	Pass
8	Linearity	R^2 > 0.995	R^2=1	Pass
9	Uncertainty	Should be calculated	Yes, 0.25 for Sample	Pass
10	Robustness	Result difference < ±6.0%	Results are within range.	Pass
11	Recovery	-	100.03	Pass

value of the T-test was 0.653, which was less than the T-tabulated values. Therefore, the method is capable of reproducing the results. The limit of Detection and Limit of Quantification of the method were 2.4 and 8.0ppm respectively. The linearity of the method was calculated for 200 to 1000ppm concentration of Clodinafop-propargyl, which showed an R^2 value of 1, exhibiting excellent performance of the method. The recovery calculation data resulted 100.030% recovery. Hence, this method of HPLC for Clodinafop-propargyl determination is capable of producing accurate and reliable results as per standard requirements.

Conclusion

After this validation experiment, as per the procedure, it is decided that the laboratory is capable of performing the clodinafop propargyl analysis by this method according to the standard. So if all protocols according to ISO/IEC 17025:2017 are followed and instruments are calibrated on an annual basis, then the method yields repeatable and reproducible results. Further, by changing temperature (i.e 30 ^{0}C and 35 ^{0}C) and by changing the Flow rate from 1.0 ml to 1.2 ml, the method gave accurate results.

Summary of Criteria

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Annexure (A)

Uncertainty Measurement (Budget)

S/N	Uncertainty Sources	Uncertainty	Type A/B	K-Factor	Uncertainty Contribution	Average or Value	Relative Uncertainty (%)	Square of Values
1	Reproducibility	0.00025829	A	1	0.0003	14.91	0.0017	3E-06
2	Analytical Balance	0.1000	B	2	0.0500	10.00	0.5000	0.25
3	Analytical Balance	0.1000	B	2	0.0500	10.00	0.5000	0.25
4	Vol. Flask	0.0100	B	1.73	0.0058	50.00	0.0116	0.000134
5	Vol. Flask	0.0100	B	1.73	0.0058	50.00	0.0116	0.000134
6	CRM	0.0050	B	2	0.0025	14.91	0.0168	0.000281
7	HPLC	0.1300	B	2	0.0650	14.91	0.4359	0.190011
Combined Uncertainty (Uc)		0.8310	@	**68 % CL**				
Confidence Level (K)		2	=	**95 % CL**				
Expanded Uncertainty (Ue)		1.6620	@	**95 % CL**				

Unit Uncertainty	0.0166
Experimental Average	14.91
Uncertainty of Experimental Value	0.25