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# METHOD DEVELOPMENT, VALIDATION AND CALCULATION OF UNCERTAINTY FOR THE DETERMINATION OF LAMBDA-CYHALOTHRIN FROM COMMERCIAL FORMULATIONS THROUGH REVERSE-PHASE LIQUID CHROMATOGRAPHIC APPROACH

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## ABSTRACT

Lambda-cyhalothrin is a pyrethroid insecticide posing an implicit curb on the pest of different crops especially cotton. The focus of the present study was the development and validation of a test procedure for the quantitative measurement of lambda-cyhalothrin from its different formulations and calculation of uncertainty. The objective of this validation method was to demonstrate its reliability for the desired application. This study was conducted at Pesticide Quality Control Laboratory, Kala Shah Kaku, Pakistan during 2019. Lambda-cyhalothrin was quantified by liquid chromatography with a diode array detector (DAD). The estimation was evaluated on agilent high performance liquid chromatograph (HPLC) equipped with C18 column using acetonitrile and water (85:15) as mobile phase. Different steps of method validation, i.e., accuracy, precision, linearity, recovery percentage, the limit of detection (LOQ) and quantification (LOD) were performed and uncertainty was calculated according to the instruction on the validation of an analytical test method. The obtained data showed that the test method was accurate (99.25% accuracy), reliable (%RSD of repeatability 0.379), precise (%RSD of reproducibility 0.30), and high recovery percentage, i.e., 102.7%. The LOQ and LOD of this test method was 0.002% and 0.01%, respectively**.** The uncertainty value of this test method for the determination of lambda-cyhalothrin (2.70%) from its formulation was observed that was, 0.016%w/w with a 5% probability level. The findings of the study validated that the method were substantiated and all the observations fall within the appropriate limit. The developed method was considerably adoptive due to its simplicity, reliability, and practicality. Hence, the proposed method had the potential to be adopted for routine analysis with ease and convenience.

KEYWORDS: Pyrethroids; uncertainty; robustness; validation; liquid chromatography; Pakistan

## INTRODUCTION

In the agriculture sector, the usage of pesticides is enormous for pest control and ultimately obtaining better crop yields globally. Annual application rate of the pesticides is 2.5 MT at a global scale and the rate is increasing constantly (Pimentel, 1995; FAO, 2002). In Pakistan, there was an abrupt increase (> 500%) in pesticide usage was observed. Besides pesticide quantity, a substantial increase was reported regarding its value during last two decades in Pakistan (Tariq, 2002). Pesticide consumption enhanced by almost 70 times (about 80%of that is being utilized on the cotton crop), however, the increase in cotton yield was only two-folds because of the adulterated and substandard pesticides. The pesticide usage in Pakistan has increased by 1169% in the previous decades and more than 10 sprays of pesticides per crop have been carried out, which is a disturbing situation. Among pesticide usage in Pakistan, use of insecticides was the highest (74%) followed by herbicides, fungicides, acaricides, and fumigants (14, 9, 2, and 1%, respectively) (Khan, 1998).

There are various groups of pesticides, including carbamates, organochlorides, organophosphates and pyrethroids. Pyrethroids possess coherent control over pests, e.g., whitefly, jassid, and thrips (Vázquez *et al.,* 2008). It was reported that about 70% of the total pesticides present in the Pakistani market was of pyrethroids (Tariq, 2002). Lambda-cyhalothrin [(*R*)-cyano- (3-phenoxyphenyl) methyl]

(1*S*, 3*S*)-3-[(*Z*)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate is a pyrethroid insecticide. It is a colorless to beige solid and has a mild odor. Lambda-cyhalothrin is non-volatile and has low water solubility. Voltage-sensitive sodium channels present in the nerves, heart, and skeletal muscle as well as cell membranes of the nervous system of pests are being connected and activated by it- that leads towards death of pest. Paralysis is initial effect of lambda-cyhalothrin on the targeted insects with little toxicity reported for birds and mammals (Wang *et al.,* 2019). Lambda-cyhalothrin is available in soluble liquid (SL), emulsifiable concentrate (EC), ultra-low volume (*ULV*) and wettable powder (WP) formulations, commercially. A toxicant is mixed with an inert material or carrier in every formulation. There are numerous reasons that affect the active ingredient concentration in agrochemical formulation- leading towards variation in actual and reported concentrations. Correspondingly, poor control of pests and crop damage may occur due to the unregulated and untested commercial products. Agricultural pesticides ordinance was implemented-with focus on the regulation of registration, standardization, distribution, sale, usage, import and production of agrochemicals in Pakistan (Mazari, 2005). Hence, in order to curb the various means of adulteration in agrochemical formulations, it seems mandatory to device an authentic and suitable method for active ingredient determination in agrochemical formulations. This will ultimately save the consumer from avoidable pest losses.

Suitability of intended procedure for a specific test is ensured by steps and process of method validation (Ermer and Miller, 2006). Method validation was defined by ISO 8402:1994 as “Confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled. Reliability of the testing is ensured by validation of test method and fitness of the intended purpose is also ensured by validation (Wood, 1999). Various analytical steps are involved in method validation to ensure its reliability and trueness for a given analytical test that is supposed to be implemented in future. The parameters of method validation include robustness, accuracy, precision, the limit of detection, linearity and the limit of quantification (Thompson *et al.,* 2002; Gul *et al.,* 2015). The reliability of the calculated results is ensured by the uncertainty in any measurement that is unavoidable. Involvements of all possible factors that can influence the results of test up to a pre-described value come under the umbrella of uncertainty (Ramsey and Ellison, 2007). Capacity determination of any test is aided by method validation and calculation of uncertainty as ISO 17025 also recommends these for any laboratory, ultimately giving solid reference for their applicability and practicability in laboratory. Hence, present study was designed with the objective of development and validation of a fast, simple and reliable HPLC-DAD procedure. That will serve the purpose of routine quality control analyses of formulated products claiming to contain lambda-cyhalothrin and uncertainty measurement calculation.

## MATERIALS AND METHODS

### Study area and year

This study was conducted at Pesticide Quality Control Laboratory, Kala Shah Kaku, Pakistan during 2019.

### Chemicals and regents

Acetonitrile, de-ionized water, mobile phase (Acetonitrile and Water: 85:15) and certiifed reference material (CRM) of lambda cyhalothrin (99.5% purity) were used. All the chemicals were of analytical reagent grade quality (ACS) or better and were purchased from Sigma Aldrich.

### Instrumentation

Agilent Technologies 1260 infinity series HPLC system with quaderinary pump and diode Array detector (DAD-VL 1260), 150 x 4.6 mm, 5 µm, 100Å packed with promosil C18 or equilent column were used. Filtering apparatus - for mobile phase, glass filtering unit with 0.45 µm filter for samples, plastic syringes with 0.45 µm filters and ultrasonic bath were also used.

### Sample preparation

Accurately weighed technical grade (CRM) of lambda-cyhalothrin having 0.05% concentration and transferred into a 50 mL volumetric flask and added 20 ml of the mobile phase. The volumetric flask was capped with a lid and sonicated in an ultrasonic bath for 10 min. After sonication made the volume up to the mark with mobile phase and then caped and mixed thoroughly. A portion of CRM filtered through a 0.45um filter into a 2 ml auto sampler vial and caped. The same procedure was followed for the sample formulation ( EC) of lambda-cyhalothrin.

### Instrument conditions of HPLC

The HPLC conditions were adjusted and maintained before standard and formulation sample run, i.e., flow rate of mobile phase (1 mL/min), oven temperature hading column ($28^0$C), wavelength of detector (235nm), injection volume (20 µL), run time (10 minutes).

### Determination of lambda-cyhalothrin

After adjusting the operating parameters, made repetitive injection (triplicate) of the lambda-cyhalothrin standard solution and then injected each test solution

in duplicate. The elution times of lambda-cyhalothrin peaks were within 5.50-6.50 min. However, column with different length and internal diameter made some variation in elution times. To calculate the final concentration of the lambda-cyhalothrin in the sample, divided the peak area of the sample with and peak area of the standard, and the weight of standard divided by the weight of sample. Then multiplied these two ratios with purity of the CRM (Dobrat and Martijn, 2009)

%age of lambda cyhalothrin = $(P_{sample} / P_{std}) \times (W_{std} / W_{sample}) \times$ purity of std

Where,

$W_{std}$ = Weight of lambda cyhalothrin standard

$W_{sample}$ = Weight of test portion/sample

Purity = Percentage purity of the lambda-cyhalothrin standard

$P_{sample}$ = Peak area of lambda-cyhalothrin from test portion

$P_{std}$ = Peak area of lambda-cyhalothrin of standard solution

**Method validation**

Following parameters were worked out for method validation.

**Accuracy**

It was a measure of how close the mean of a finite number of results were to the true value. To determine the accuracy of the method, the data obtained from the repeatability of two analysts was used and it was calculated by using the following formula (Desta and Amare, 2017)

**%** Accuracy= 100- Error (%)

While

$$Error\ \% = \frac{Mean\ value\ (x) - Actual\ value\ (A)}{Actual\ value\ (A)} \times 100$$

According to CIPAC (1999) a good validated method must have accuracy > 85%.

**Precision**

It is a measure of how close results are to one another. In precision, we determined two things repeatability and intermediate precision. For the determination of repeatability of the method, only one analyst prepared samples of known concentration and run on the high performance liquid chromatography (HPLC) to take 10 repeats of the sample**.** Relative standard deviation (RSD) was calculated of these repeats at the end**.** Likewise, for the assessment of intermediate precision of the method, two analysts prepared10 samples of the same concentration separately and run on the HPLC, one just after the other, to take 10 repeats (Kalra, 2011).

**Linearity and range**

Range of concentrations from 0.0025% to 0.0500% with regular interval was used to determine the linearity of the method. For this purpose, a working standard solution of concentration 0.1% in 100 mL volumetric flask was prepared and further concentrations (0.0025%, 0.0075%, 0.0125%, 0.0250% and 0.0500%) were prepared by using the same stock solution.

**Limit of quantification (LOQ)**

Using the linear equation of the method, from the linearity study, LOQ was calculated from the following formula (Jiang *et al.,* 2004; Knoll, 1985);

LOQ = 10 × y-intercept/ slop

**Limit of detection (LOD)**

Using the linear equation of the method, from the linearity study, LOQ was calculated from the following formula (Jiang *et al.,* 2004; Knoll,1985);

*LOD= 03 × y-intercept/ slop*

**Recovery (%)**

Recovery studies were performed with the sample prepared at concentration level 0.0025%, 0.0075%, 0.0125%, 0.0250% and 0.0500%. Recovery was calculated using formula (Wood,1999; Standard,2006)

Recovery (%) = $[ (C_d)/C_m] \times 100$

Where;

$C_d$ = analyte concentration determined
$C_m$ = analyte concentration determined from the blank

**Robustness**

The evaluation of the robustness of the method was carried out by altering various parameters of the test method (Padmasubashini *et al.,* 2020). In this study, we changed column temperature (changed from 28°C to 30°C and flow rate of mobile phase (changed from 1 ml $mint^{-1}$ to1.2 ml $mint^{-1}$) and then checked the recovery and RSD.

**Uncertainty**

The Eurachem guide (ISO and OIML, 1995) was used to calculate the uncertainty of this test method.

Uncertainty in the results might occured due to the certain factors, most often analyst, method, chemical / solution, environment, etc. All factors constitute the function of combined uncertainty. The calculation of total uncertainty of all factors is called the budget of uncertainty, for each of the test separate uncertainty budget will be calculated. The square root of sum of squares of all factors was called the combined uncertainty. It was the uncertainty of system at 68% of confidence level (C.L).

$$UC = \sqrt{(U_{(x\,1)})^{2} + (U_{(x\,2)})^{2} + (U_{(x\,3)})^{2} + (U_{(x\,4)})^{2}}$$

Whereas, UC = Combined uncertainty

The standard ISO 17025:2017 required the labs to express their estimated uncertainties with certain confidence called expanded uncertainty. This confidence were expressed through using Confidence Level which was more commonly known as *k* factor

Expanded uncertainty = Combined uncertainty x confidence level (k)

Expended Uncertainty (Ue) = Uc x K

Whereas, confidence level, *k* value was 2 for 95% C.L. and 3 for 99.7% C.L.

## RESULTS AND DISCUSSION

### Method validation

### Accuracy of the test method

It is a measure of the closeness of the mean of finite number of results to the true value. This is sometimes termed as trueness (Desta and Amare, 2017).The accuracy of this test method for the determination of lambda-cyhalothrin was 99.25% (Table1). This high value of accuracy showed that this test method was reliable for the determination of lambda-cyhalothrin from the different pesticide formulations. Further, the accuracy of this test method also fulfilled the criteria of CIPAC (1999) for accuracy of the validated method, i.e., accuracy must be >85% for a well-validated method.

### Precision of the test method

It is a degree of closeness of results with each other, or closeness of agreement (degree of scatter) defines the precision of an analytical procedure among a series of measurements acquired from same homogeneous sample through multiple sampling following the prescribed conditions. The precision consists of two parts, i.e., repeatability and reproducibility (intermediate precision) (Kalra, 2011). The RSD of repeatability, i.e., 0.379% (Table 2) was much less than the acceptable limit of AOAC (Latimer, 2019), i.e., 3%. Similarly, the RSD of intermediate precision, i.e., 0.30% (Table 2) was also much less than the acceptable limit set in AOAC for a well-validated method (Latimer, 2019), i.e., 3%. These results of precision showed that this method has the ability to produce precise results with good accuracy for the determination of lambda cyhalothrin from different pesticide formulations.

### Linearity and range of the test method

The ability (within prescribed range) of an analytical procedure to acquire test results in such a way that there must be a directly proportional relation between results and analyte concentration present in the sample is termed as linearity(Wood,1999). The linearity graph of this test method showed there was a strong correlation between peak area and concentration of lambda-cyhalothrin (Fig. 1 and 2). The correlation coefficient ($R^2$) value was 0.9997, and it was higher than the acceptable limit, i.e., $R^2$>0.998 set in CIPAC for a well-validated method (CIPAC, 1999).

**Table 1. Accuracy of the test method for the determination of lambda cyhalothrin from commercial formulations**

| No. of readings | Analyst 1st readings | Analyst 2nd readings | Mean of analyst 1 & analyst 2 | Analyst 1 | | Analyst 2 | | Mean of analyst 1 and analyst 2 | | Pass/fail |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | % Error | % Accuracy | % Error | % Accuracy | % Error | % Accuracy | |
| 1 | 0.0501 | 0.0495 | 0.0498 | 0.77 | 99.23 | 0.73 | 99.27 | 0.75 | 99.25 | Pass |
| 2 | 0.0495 | 0.0495 | 0.0495 | | | | | | | |
| 3 | 0.0496 | 0.0497 | 0.0496 | | | | | | | |
| 4 | 0.0494 | 0.0494 | 0.0494 | | | | | | | |
| 5 | 0.0496 | 0.0496 | 0.0496 | | | | | | | |
| 6 | 0.0497 | 0.0497 | 0.0497 | | | | | | | |
| 7 | 0.0496 | 0.0496 | 0.0496 | | | | | | | |
| 8 | 0.0497 | 0.0496 | 0.0497 | | | | | | | |
| 9 | 0.0496 | 0.0502 | 0.0499 | | | | | | | |
| 10 | 0.0494 | 0.0495 | 0.0495 | | | | | | | |
| Mean (X) | 0.0496 | 0.0496 | 0.0498 | | | | | | | |

**Table 2. Precision of the test method for the determination of lambda cyhalothrin from commercial formulations**

| Parameter | No. of observations | Analyst 1 (Measured con. %) | Analyst 2 (Measured con. %) | Acceptable limit | Pass/fail |
|---|---|---|---|---|---|
| Intermediate precision | 1 | 0.0501 | 0.0495 | (AOAC) RSD (%) <3% | Pass |
| | 2 | 0.0495 | 0.0495 | | |
| | 3 | 0.0496 | 0.0497 | | |
| | 4 | 0.0494 | 0.0494 | | |
| | 5 | 0.0496 | 0.0496 | | |
| | 6 | 0.0497 | 0.0497 | | |
| | 7 | 0.0496 | 0.0496 | | |
| | 8 | 0.0497 | 0.0496 | | |
| | 9 | 0.0496 | 0.0502 | | |
| | 10 | 0.0494 | 0.0495 | | |
| | Mean (N=10) | **0.0496** | **0.0496** | | |
| | Mean (N=20) | 0.0496 | | | |
| | SD of N=20 | 0.00015 | | | |
| | RSD % of N=20 | 0.30 | | | |
| Repeatability | **No. of observations** | **Result (measured Con. %)** | | **Acceptable limit** | **Pass/fail** |
| | 1 | 0.0501 | | (AOAC) RSD (%) <3% | Pass |
| | 2 | 0.0495 | | | |
| | 3 | 0.0496 | | | |
| | 4 | 0.0494 | | | |
| | 5 | 0.0496 | | | |
| | 6 | 0.0497 | | | |
| | 7 | 0.0496 | | | |
| | 8 | 0.0497 | | | |
| | 9 | 0.0496 | | | |
| | 10 | 0.0494 | | | |
| | Mean (N=10) | 0.0496 | | | |
| | SD | **0.00019** | | | |
| | | | | | |
| | RSD % | 0.379 | | | |

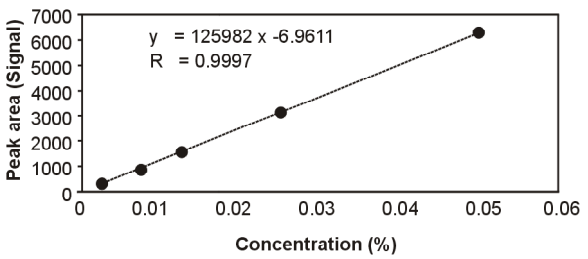


**Fig. 1. Linearity graph showing correlation between peak area and concentration of lambda-cyhalothrin**

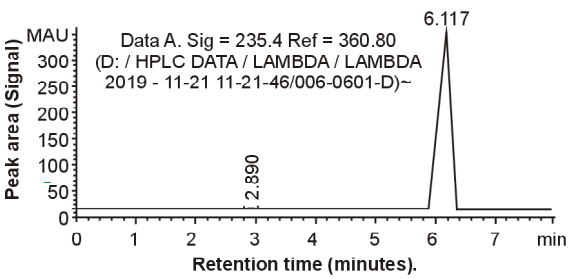


**Fig. 2. Chromatogram of lambda-cyhalothrin (0.05%) showing peak area of retention**

## Detection and quantification limits of the test method

The limit of detection (LOD) of the method is that the minimum amount can be observed by employing the method, however, it cannot be quantified accurately. The limit of quantification (LOQ) of the method is the minimum amount that can be detected and quantified accurately by using the method (Jiang *et al.,* 2004; Knoll,1985). The LOD and LOQ of this method for the determination of lambda-cyhalothrin were calculated by using the linear equation of linearity graph, and their values were 0.002 and 0.01%, respectively (Table 3).

## Recovery percentage of the test method

Percentage recovery values were deliberated by comparing concentrations appraised from the spiked supporting electrolyte with added concentrations of lambda-cyhalothrin. Exemplary values of the recoveries, i.e. 120.45, 94.72, 97.95, 100.34 and 100.11%, for 0.0025, 0.0075, 0.0125, 0.025 and 0.0500% of lambda-cyhalothrin, respectively, established the accuracy of the proposed method and provided the promising confirmation that the laborated method might be

**Table 3. Recovery percentages of the test method after spiking different concentrations of lambda cyhalothrin**

| Sr. No. | Spiking level (A) | Experimental value (X) | X/A | Recovery (%) | Mean recovery (%) | Acceptable criteria | Pass/fail |
|---|---|---|---|---|---|---|---|
| 1 | 0.0025 | 0.00301 | 1.2045 | 120.45 | 102.716 | 70-120% (CIPAC, 1999) | Pass |
| 2 | 0.0075 | 0.00710 | 0.9471 | 94.72 | | | |
| 3 | 0.0125 | 0.01224 | 0.9794 | 97.95 | | | |
| 4 | 0.025 | 0.02508 | 1.0034 | 100.34 | | | |
| 5 | 0.05 | 0.05005 | 1.0011 | 100.11 | | | |

employed in the analysis of real samples. Further, all the recoveries (%) values, as well as mean recovery value (Table 3) full, filled the acceptable percentage recovery criteria of a well-validated method of CIPAC (1999), i.e. recovery percentage should be 70-120% (Durovic *et al.,* 2016).

## Robustness of the test method

Robustness is the comparison of the actual method with some modification or variation in the method. The results of modification in methods are compared to different forms, e.g., RSD and recovery percentages. (Nazir *et al.,* 2020). The data obtained after the modification in column temperature and mobile flow rate summarized in Table 4.

**Table 4. Robustness data of the test method for the determination of lambda-cyhalothrin from pesticide formulations**

| Method parameter | Parameter condition | RSD (%) | Recovery (%) |
|---|---|---|---|
| Column temperature (°C) | 28°C | 0.246 | 99.718 |
| | 35°C | 0.895 | 99.126 |
| Flow rate (mL/minute) | 1 ml $mint^{-1}$ | 0.088 | 99.835 |
| | 1.2 ml $mint^{-1}$ | 0.194 | 98.588 |

The data of modification in column temperature showed that recovery (%) of the two variables was lying within the acceptable limit. However, RSD (%) was found to significantly less using the column temperature 28 °C. So, the column temperature of 28 °C was hereby recommended. For the flow rate parameter, the RSD (%) of two variables was lying within the acceptable limit. However, RSD (%) was found to very less in case of 1 ml/mint as compared with 1.2 ml/mint. Similarly, recovery (%) of the two variables are lying within the acceptable limit. However, Recovery (%) was found to be very close to 100% in case of 1 ml/mint as compared with 1.2 ml/mint. So, 28 °C column temperature and 1 ml $mint^{-1}$ flow rate produced reliable results.

## Uncertainty of the test method

The concluded value may resonate in a range that is called uncertainty. There exist the positive and negative forms of uncertainty (Padmasubashini *et al.,* 2020). The final uncertainty budget was determined by the calculation and combination of these both types of uncertainty sources. Standard deviation's reproducibility and repeatability was employed to calculate the Type A, that was further used for the measurement of standard uncertainty. While, Type B was calculated through following the various distribution laws, e.g., rectangular distribution, normal distribution, and triangular distribution. The relative uncertainty calculation was carried out by employing uncertainty budget. The expanded uncertainty having a certain confidence level was obtained from the multiplication of combined uncertainty results along relative uncertainty with coverage factor. The test method's uncertainty value was deliberated as 0.01648943%w/w (95% confidence level) (Table 5).

## CONCLUSION

The method fulfilled all the parameters for a well-validated method according to standard guidelines. Further, the suggested method was established to be simple, accurate, precise, linear and robust for the

**Table 5. Uncertainty of the test method for the determination of lambda-cyhalothrin from pesticide formulations**

| Uncertainty Budget of Lambda Cyhalothrin (2.70%) | | | | | | |
|---|---|---|---|---|---|---|
| Sr. No. | Sources of uncertainty | Type A/B | Uncertainty contribution | Sensitivity co-efficient | Relative uncertainty | Combining uncertainty |
| 1 | Uncertainty due to precision ($U_{Prec}$) | A | 0.001727382 | 1 | 0.001727382 | 2.9839E-06 |
| 2 | Uncertainty due to recovery ($U_{recv.}$) | A | 3.18434E-05 | 1 | 3.18434E-05 | 1.014E-09 |
| 3 | Uncertainty due to the purity of lambda cyhalothrin CRM ($U_{PCRM}$) | B | 0.002512563 | 1 | 0.002512563 | 6.313E-06 |
| | | | | | SQRT(SUM(I5:I8) | 0.00304924 |
| | Mean concentration from precision study (%) | | 2.703862571 | | | |
| Combined uncertainty (%) | | | 0.00824471 | @ | 68 % CL | |
| CL (K) | | | 2 | = | 95% CL | |
| Expanded uncertainty (%) | | | 0.01648943 | @ | 95% CL | |

determination and quantification of lambda-cyhalothrin. consequently, the method proved to be easy and convenient for adaptation in routine analysis.

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## CONTRIBUTION OF AUTHORS

| Sr. No. | Author's name | Contribution | Signature |
|---|---|---|---|
| 1. | Zeshan Aslam | Conceived the idea and conducted the research |  |
| 2. | Muhammad Aslam Avais | Provided guidelines during research work |  |
| 3. | Muhammad Rashid Farooq | Helped in manuscript write-up |  |
| 4. | Muhammad Adnan Rafique | Worked as analyst |  |
| 5. | Muhammad Zia Ul Haq | Proof read the manuscript |  |
| 6. | Arslan Nazarat | Worked as alternate analyst |  |
| 7. | Ali Afzal | Conducted statistical analysis |  |
| 8. | Muhammad Awais Khalid | Helped in calcualting uncertainty |  |