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Short communication

Determination and quality monitoring of naphthyl acetic acid in commercial plant growth regulator formulations using HPLC technique

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Abstract

Plant growth regulators are useful for promoting plant growth and their consumption in horticultural crops is increasing day by day. However, their quality and purity are most important characteristics to estimate their efficacy. In Pakistan, the determination and quality monitoring of naphthyl acetic acid (NAA) in commercial plant growth regulator formulations by using HPLC technique is very limited. For the purpose of registration of plant growth regulators (PGRs) for marketing and for post-registration quality monitoring, a robust, simple, and accurate analytical method is needed. In this study, a high performance liquid chromatography (HPLC) method for the determination of NAA in commercial PGR samples was validated under the laboratory conditions and available resources of Provincial Reference Fertilizer Testing Laboratory (PRFTL) Raiwind, Lahore. The guidelines and values set by Collaborative International Pesticides Analytical Council (CIPAC) were considered as standard for method evaluation. The chromatographic conditions of HPLC method under study are comprised of (i) mobile phase; mixture of acetonitrile: water (30:70,v/v); (ii) C8 column; (iii) UV detection at 255nm; (iv) Flow rate of 1.0mL / minute; (v) and injection volume of 10 µL. The method demonstrated clear response linearity with correlation coefficient (r) of 0.999. In order to determine the precision, an interlaboratory comparison of NAA was conducted and percent relative standard deviation (RSD) was calculated from results received from eight (8) participating laboratories. The Horwitz equation was followed to evaluate precision of method. The experimental inter-laboratory RSD (RSD_R =0.168) was within the Horwitz acceptable limit of RSD_R (2.097). Method accuracy was evaluated by calculating percent recovery of analytical standard of NAA obtained from Sigma Aldrich along with its certificate of analysis. The recovery of NAA standard ranged between 99.96 to 100%. The results revealed that aforementioned HPLC method fulfilled the validation criteria of CIPAC and can be followed under laboratory conditions of PRFTL, Raiwind, Lahore for routine analysis of NAA in commercial PGRs. Additionally, the quality of commercial NAA samples marketed by different companies was assessed by following aforesaid validated HPLC method. Ten samples of NAA were collected from market and were analyzed. The results were tested for fit or unfit and compared with the company/manufacturers' claim considering the decision rule followed by the Agriculture Department, Government of Punjab, Pakistan. The NAA contents in samples ranged from 4.20 ± 0.10 to 5.13 ± 0.05%. The results showed that all the samples were fit, and it can be inferred that presently the quality of commercial NAA marketed in district Lahore is satisfactory.

Keywords: Naphthyl Acetic Acid; plant growth regulators; precision, accuracy; method validation

There is an increasing trend of using PGRs for growing vegetable crops and viticulture. These PGRs are either synthetic or have been derived from natural source and influence the developmental or metabolic activities

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in plants when used in low concentration. The developmental processes which can be controlled using PGRs include seed germination, dwarfism, shortening of dormancy span, initiation of flowering and fruiting (Rademacher, 2015). The higher doses of auxin related PGRs may give opposite effects such as defoliation and stunted growth. Hence, these auxin-related PGRs were used as defoliant during military operations in Vietnam War (Stellman *et al.,* 2003).

Naphthyl acetic acid (NAA; $C_{12}H_{10}O_2$) belongs to auxin related growth regulator (Yamamoto and Yamamoto, 1998) and primarily involved in cell division. Therefore it is used to stimulate rooting in cuttings of many trees, shrubs, and vines (Cobb and Reade, 2010). Its biochemical effect on increasing chlorophyll content, nitrogen level and oil contents of mustard plant has also been reported (Begum *et al*., 2018).

Different manufacturers in Punjab province are marketing their NAA products and there is a need to validate a rapid HPLC method for quality monitoring of NAA products. The available HPLC method for analysis of NAA employs Photodiode-array detector (PDA) and specific Discovery HS-C18 5µm column with the retention time of ~11 minutes (Sigma-Aldrich, 2021). This method was partially modified under the laboratory conditions and available resources of PRFTL, Lahore (Table 1) and afterwards, method was validated following standards of CIPAC.

The efficacy of commercial NAA depends on its quality and purity. In Pakistan, the determination and quality monitoring of NAA in commercial plant growth regulator formulations by using HPLC technique is very limited. This method validation was conducted with the objectives to (i) evaluate the modified HPLC methodology for its suitability for routine quality control analysis of commercial NAA and (ii) monitoring the quality of commercial-NAA PGR being marketed in the Lahore district.

The quality testing needs special care and certified reference materials (CRM). The analytical standard for NAA (95.9%; CAS. No. 86-87-3 and Batch No. BCBW4206) was obtained from Sigma-Aldrich Laborchemikalien GmbH, along with Certificate of Analysis (CoA). The stock solution was prepared from CRM and then working standard solutions of NAA were prepared in three replicates at four concentration levels typically, 250, 500, 750 and 1000 mg NAA L^{-1}. To determine the repeatability, three replicates of each concentration level were injected to get the average peak area of each concentration level. These peak area values were used to develop the standard calibration curve. The correlation coefficient (r), slope and intercept were calculated using R language (Ihaka and Gentleman, 1996) with confidence interval at 95%. The criteria for response linearity (correlation coefficient ≥ 0.997) set by CIPAC (1999) was followed for evaluating response linearity.

Table 1: Chromatographic Conditions for analysis of NAA in commercial PGRs

Chromatographic Condition	Sigma-Aldrich*	Modified at PRFTL Lahore
Mobile phase	30% Acetonitrile, 70% Water + 0.1% Phosphoric Acid	30% Acetonitrile, 70% Water
Flow	1.8ml/min	1.0ml/min
Column	Discovery HS-C18 L=150mm, ID= 4.6mm Particle size=5µm	Brownlee Analytical (Perkin Elmer) C8 L=250mm, ID=4.6mm Particle size=5µm
Retention	~10.98 minutes	~2.8 minutes

Ten commercial PGR samples containing NAA as an active ingredient were collected from market. The HPLC grade solvents were used for analysis. Sample solutions were prepared by dissolving appropriate quantity of commercial NAA in mobile phase and diluted in such a way so that its concentration falls within the range of standard calibration curve.

The HPLC system used in this study include LC autosampler, solvent manager, column oven and UV/VIS detector (Perkin Elmer, USA, Flexar series). The column used was Brownlee™ analytical, C-8 (4.6 mm i.d x 250 mm, 5micron) Perkin Elmer, USA. The system has valid calibration status obtained from Pakistan National Accreditation Council (PNAC) accredited calibration laboratories (PCSIR, Lahore, Lab. No. 002 and AIMS, Lahore, Lab. No. 131). The licensed, Perkin Elmer provided software "Chromera" was used to control the instrument. The column temperature was set at 25 ˚C during all runs.

Table 2: Interpretation of z-score

z-score	Interpretation
$Z \leq 2.0$	Satisfactory results
$2.0 < Z < 3.0$	Questionable results
$Z \geq 3.0$	Unsatisfactory results

Table 3: Calibration curve – data sheet for naphthyl acetic acid

NAA (mg L^{-1})	Peak area			
	Replicate-1	Replicate-2	Replicate-3	Peak area (Mean)
Blank	Autozero	Autozero	Autozero	Autozero
250	1708793	1708788	1708780	1708787
500	3392745	3392749	3392740	3392745
750	5130491	5130502	5130480	5130491
1000	7126839	7126844	7126835	7126839
Equation for regression line = y = 6985.7X			Correlation coefficient (R^2) = 0.999	

Table 4: Linear regression parameters of calibration of naphthyl acetic acid-Plant Growth Regulator

Slope	Intercept	r	Concentration (Range)
6985.7	-63304	0.999	250 - 1000 mg NAA L^{-1}

Table 5: Z-score of inter-lab comparison of naphthyl acetic acid

Lab/sample Code	Lab Name	NAA (w/v %)	Average	Std. Deviation	Z-Score
PRFTL-NAA- 2	Pak China Chemicals, Lahore	5.49	5.17	0.168	1.90
PRFTL-NAA- 4	Exin Chemicals, Multan	5.07	5.17	0.168	-0.60
PRFTL-NAA- 5	Hexon Chemicals, (Pvt.) Ltd. Multan	5.08	5.17	0.168	-0.57
PRFTL-NAA- 6	PCSIR* Laboratories, Lahore	5.10	5.17	0.168	-0.42
PRFTL-NAA- 7	Solex Chemicals, Multan	5.11	5.17	0.168	-0.38
PRFTL-NAA- 10	Warble (Pvt.) Ltd. Multan	5.06	5.17	0.168	-0.65
PRFTL-NAA- 11	PRFTL, Raiwind**	5.38	5.17	0.168	1.25
PRFTL-NAA- 12	Buraq Agro Chemicals, Multan	5.07	5.17	0.168	-0.60

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Table 6: Analysis results of ten (10) commercially marketed NAA-PGR products

Description	Rep-1	Rep-2	Rep-3	Average (%w/v) ± SD	Status
Company No. 1	4.20	4.10	4.30	4.20 ± 0.10	Fit
Company No. 2	4.65	4.50	4.65	4.60 ± 0.09	Fit
Company No. 3	4.20	4.15	4.25	4.20 ± 0.05	Fit
Company No. 4	4.35	4.20	4.40	4.32 ± 0.10	Fit
Company No. 5	4.45	4.36	4.28	4.36 ± 0.08	Fit
Company No. 6	4.10	4.21	4.35	4.22 ± 0.12	Fit
Company No. 7	5.10	4.90	5.00	5.00 ± 0.10	Fit
Company No. 8	5.08	5.17	5.15	5.13 ± 0.05	Fit
Company No. 9	4.97	4.65	4.70	4.77 ± 0.17	Fit
Company No. 10	5.00	4.95	5.05	5.00 ± 0.05	Fit

The analysis was performed following isocratic method using mixture of Acetonitrile-Water (30:70,v/v) as a mobile phase and UV detection was performed at 255 nm. The flow rate was adjusted at 1.0 mL/min . The injection volume was set at 10 µL.

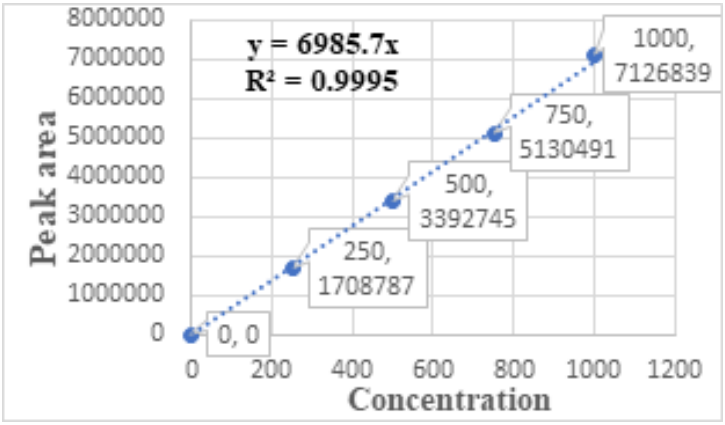

Figure 1: Calibration curve between concentration and peak area of naphthyl acetic acid. The correlation coefficient (R^2 = 0.999) represents good predictability of dependent variable

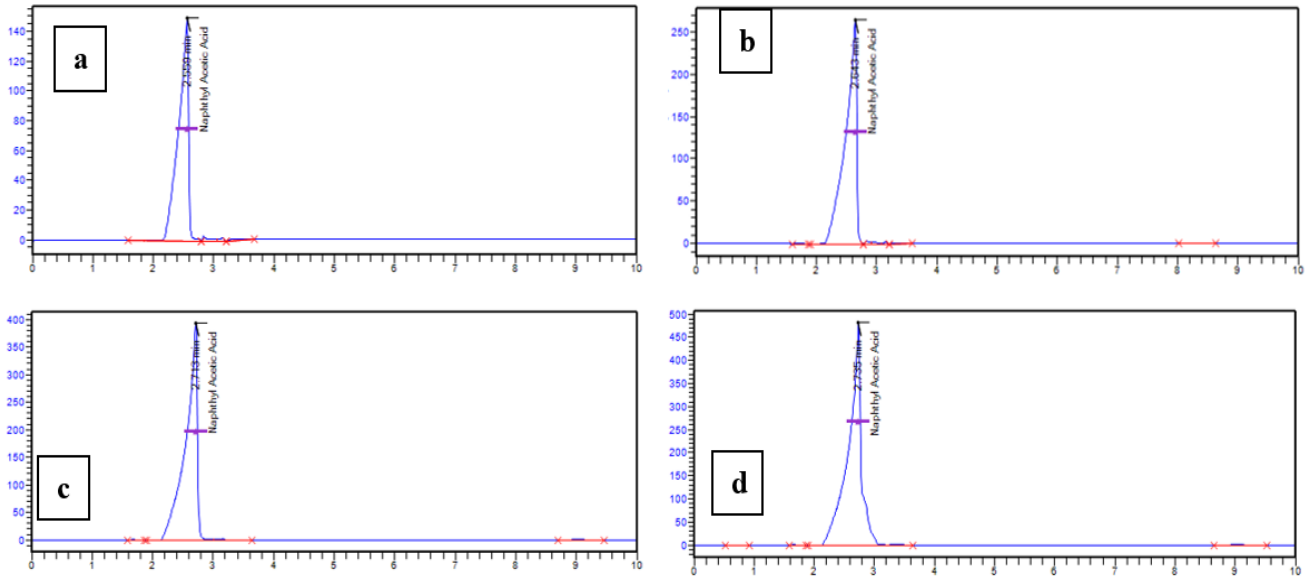

Figure 2: Chromatograms of NAA (a) 250 mg NAA L^{-1}, (b) 500 mg NAA L^{-1} (c) 750 mg NAA L^{-1} and (d) 1000 mg NAA L^{-1}

The precision of HPLC method under study was determined by conducting inter-laboratory comparison of commercial NAA. One coded liquid sample of NAA was sent to two public and six private sector laboratories along with the aforesaid HPLC method for analysis. The results received from participating laboratories were used to calculate the Z-Score of each laboratory. Generally, Z-Score is used for evaluating the results of Inter-Lab Comparison (ILC) and Proficiency Testing (PT). It represents the overall performance of the laboratory and depends upon type of analysis, analytical method used and competency of analyst (DEMIRCIOGLU and KARAPINAR). It is a numerical measurement of a value's relationship to the mean in a group of values. The Z score was calculated using following formula.

$$\text{Z Score} = \frac{xi - X}{\sigma}$$

xi = Results of lab. i

σ = Standard Deviation

X = Assigned value. It was determined by taking average of results obtained from all participant

laboratories. The results that have one of the following issues were excluded from calculation.

i. Having wrong units
ii. Non-numerical results or a range of value
iii. The result that is 10 times greater or smaller than the majority of submitted results (reporting error).

The criteria used for interpreting Z-score is given in table-2. The Z-Score value in range of ± 2.0 is classified as satisfactory. The Z-Score value equals or above ± 3.0 range indicates unsatisfactory results and needs to take necessary corrective action to deal with the problem.

All the data obtained was statistically analyzed to determine descriptive statistics such as mean, standard deviation, linear regression, and correlation coefficient. The open source R 286 statistical package was used for statistical analysis (Ihaka and Gentleman, 1996).

The performance of analytical method under study was evaluated on the basis of (1) determination of response linearity for the analyte under study, (2) determination of method precision, and (3) estimation of method accuracy by calculating percent recovery of analytical standard and its comparison to its certified reference value (CIPAC, 1999).

Linearity of an analytical method represents its capacity to give test results in proportionate to the concentration of analyte in test material up to a certain range of concentration (APVMA, 2014). The visual observation of response in relation to concentration of analyte illustrated a clear linear correlation up to analyte concentration of 1000 mg NAA L^{-1}. This depicts that method performance is excellent. Linear trend in calibration curve has good behavior and predictability (R^2 = 0.999) which showed good predictability of dependent variable (Table-3, Figure-1 & 2). The correlation coefficient, slope and intercept values qualify the CIPAC (1999) criteria (Table-4).

Precision describes the random errors in method. It is generally represented in terms of repeatability or reproducibility of procedure. The precision of HPLC test method for commercial NAA was evaluated from the ILC result received from eight (8) participant laboratories. The concentration of NAA in commercial samples ranged from 5.06 to 5.49 % NAA (w/v) with mean value of 5.17 and standard deviation of 0.168. These results were evaluated on the basis of Z-score. The Z-score of all the participating laboratories was within safe limit of ± 2.0 which indicated the acceptable repeatability and reproducibility of HPLC method for routine analysis of NAA (Table 5).

The reproducibility data was evaluated by using Horwitz equation (Boyer *et al.,* 1985).

$$RSD_R = 2^{(1 - 0.5 \log C)} \quad \text{(equation 1)}$$

Where RSD_R is the inter-laboratory relative standard deviation and C represents the concentration of analyte in sample represented in decimal fraction (Karasali and Ioannou, 2009). The results were accepted considering the modified Horwitz equation (CIPAC, 1999).

$$\text{Acceptable } RSD_R < 2^{(1 - 0.5 \log C)} \times 0.67 \quad \text{(equation 2)}$$

The RSD_R value calculated using Horwitz equation is 3.130 (equation 1) whereas the acceptable limit for Horwitz value for RSD_R is 2.097 (equation 2). The experimental RSD_R value obtained from inter-laboratory comparison is 0.168 which falls within the acceptable limit of Horwitz equation.

Accuracy is the closeness of measure to its true value. The analytical grade standard of NAA obtained from Sigma Aldrich was analyzed for ten times (n=10) and analytical results were compared with standards' true value mentioned in its certificate of analysis (CoA). The CIPAC (1999) criteria for % recovery (97.0 to 103.0) was used to evaluate method accuracy.

The percent recovery of analytical standard of NAA following aforesaid HPLC method ranged from 99.96 to 100.0% with mean value of 100 and relative standard deviation (%RSD) of 0.033. The results showed that method is accurate.

Ten samples of commercially available NAA products of different companies were analyzed following the above-mentioned validated method. The company and brand names of products were kept anonymous for the sake of confidentiality. The analysis results were compared with the manufacturer's / company claim and were categorized considering the decision rule of PRFTL for test results. The decision rule states that the 5% of claim value, including measurement uncertainty, will be taken as tolerance limit, and will be considered during reporting of test results. The sample will be declared as "unfit" if the sum of test results and tolerance limit is less than the claim value.

The results of present study showed that all the samples fall within the Fit category (Table 6). Generally, overall quality of marketed NAA-PGR is satisfactory in district Lahore.

The HPLC method presented in this research article was evaluated and validated for quality testing of NAA under the laboratory conditions of Provincial Reference

Fertilizer Testing Laboratory, Raiwind, Lahore (Accreditation Lab. No. 128). The study concluded that method is simple, accurate and fast, because its' Retention time ~ 2.5 minutes is very short. It can be used for routine quantitative analysis of naphthyl acetic acid in commercial plant growth regulators.

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