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ESTABLISHMENT AND VALIDATION OF A RELIABLE GRAVIMETRIC METHOD FOR HUMIC ACID ESTIMATION

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Abstract
Fertilizer samples are routinely analyzed to determine their nutrient composition; however, variations in results often arise due to differences in analytical methods. Therefore, the primary objective of this study was to develop and validate a gravimetric method for the determination of humic acid. The method was validated at the Soil and Water Testing Laboratories (SWTL), accredited under ISO 17025, D.G. Khan. Validation parameters included repeatability, reproducibility, limit of detection (LOD), limit of quantification (LOQ), recovery, and bias. Descriptive statistics such as mean, standard deviation, and relative standard deviation (RSD) were employed, while reproducibility was assessed using the t-test. The LOD and LOQ were determined to be 0.145% and 0.484% humic acid, respectively. The method exhibited excellent precision, with a repeatability RSD of 0.4725% and a calculated t-value (0.05) lower than the tabulated threshold (2.262) at a 5% significance level (95% confidence interval). The recovery rate of humic acid was 102.34%. Z-scores from QUATEST3 (Vietnam) were within the acceptable range, and a strong correlation (r = 0.999) was observed between the true and calculated values, confirming the method's reliability. Overall, all validation parameters met the required standards, demonstrating that the developed gravimetric method is accurate, precise, and suitable for routine determination of humic acid in fertilizers.

INTRODUCTION

Lignin, tannins, cellulose, cutins, and other degraded plant and animal components are examples of humic compounds (Tan et al., 2000; Billingham, 2012; Hayes and Swift, 2020). After adding harvested leftovers, the soil has high levels of HS (Wiesler et al., 2016). Most arable land now has less harvested residues due to increased livestock and biogas production, which lowers the amount of HS in the soil. Researchers have tried to use external applications to make up for the lost HS over the past few decades (Rose et al., 2014; Gerke, 2018). Soil, coals, lignites, as well as organic contents, are the primary commercial sources of HS (Gollenbeek and Van Der Weide, 2020; Yang et al., 2021). According to their ability to be soluble in different solutions (i.e acidic, alkaline solutions), they are classified as fulvic acid and humic acid (De Melo et al., 2016). Because the humin percentage in HS does not decompose, scientists have concentrated on the humic acid fraction, as well as on fulvic acid fractions, as they can quickly increase soil fertility and health. According to Among et al., humic substances on crop productivity play an advantageous part in soils as well as plants because the humic acid fractions and Fulvic Acid fractions of humic materials are more reactive chemically and resistant to microbial responses (Billingham, 2012). Because of their amphiphilic characteristics and long-term degradation resistance, HA can form very complex cations (Wood, 1996).

Around 60% of the HA fraction is organic carbon (C), which is crucial for soil microbial growth (Sible et al., 2021). It also contains sulphur (S), hydrogen (H), oxygen (O), and nitrogen (N) in addition to C.

For example, humic acids can improve the texture of soil, soil structure as well as microbial growth of soil, increasing its physic ochemical properties (Fuentes et al., 2018; Shah et al., 2018); enhance the availabilty of different nutrient elements in soil, particularly differentmicronutrient through chelating andtransportationof micronutrientsin theplant (Yang et al., 2021); and causesprecipitationofpoisonous heavy metal contents and decrease their transport to plants in turn lowers the amount of toxic substances that plant consume (Wu et al., 2017). By boosting plant growth-promoting hormones like auxin as well as cytokinin that support photosynthesis, nutrient breakdown, and develop stress resistance, humic acids also stimulate the growth of crops (Billingham, 2012; Rose et al., 2014; Canellas et al., 2020; Laskosky et al., 2020; Nardi et al., 2021; van Tol de Castro et al., 2021). Following HA treatment, earlier work has also found no impacts on soil health and the growth crop (Bybordi and Ebrahimian, 2013; Bassiouny et al., 2014; Mukherjee et al., 2014; Kelapa and Banyuasin, 2016). Higher Humic acid dosages are linked to improved physical properties of soil (Gollenbeek and Van Der Weide, 2020), but it is still unclear how they will affect crops and soil chemical properties (Rose et al., 2014).

By standard, the objective of validation of the analysis protocol is to guarantee that it accomplishes the appropriate criteria. Present research aimed to develop and validate a gravimetric technique for determining humic acid in various fertilizers.

of precision, repeatabilty, reproduciblity, limit of detection and quantification limits, percent recovery as well as for bias (Guideline, 2007; Sahoo et al., 2018). The present study regarding the validation of the method was conducted at the Soil & Water Testing Lab. Dera Ghazi Khan District of Punjab, Pakistan (Pakistan).

Accuracy

By definition, accuracy is referred to as "closeness of results to the actual result". For determining the accuracy of any method/protocol, the resulting data regarding of repeatability of two dissimilar scientists was used. According to Collaborative International Pesticides Analytical Council (CIPAC 1999), the better developed/validated protocol has a percent accuracy greater than 85.0 %. The accuracy was calculated using the method of (Desta and Amare (2017) and Sinshaw et al. (2019)).

Accuracy (%) = 100 – error

Precision

Precision is the "agreement between a set of replicated measurements without having any information of actual values". For the determination of the precision, the obtained results from the repeatability as well as the reproducibility were applied. For the repeatability of the first analyst (analyst-1), ten samples of humic acid were arranged with the same concentration of humic acid and their active ingredients were measured. Nevertheless, for the reproducibility of the second analyst (analyst-2), the humic acid samples of similar concentrations were prepared and analysed by taking 10 repeated readings (Barnawal et al., 2016).

Limit of detection and limit of quantification

Detection limits (LOD) are defined as the lowest quantity of a material which can be certainly detected as well as distinguished from zero (0). Nonetheless, it cannot certainly be quantified (González et al., 2018; McDowall, 2005). Whereas, the quantifying limits (LOQ) are the lowest quantity of the material which could be determined quantitatively with a satisfactory range concerning precision and accuracy (González et al., 2018; González & Herrador, 2007; Markley et al., 1998).

Measurement of uncertainty

For uncertainty determination, the Eurachem Guide was consulted. The uncertainty in the outcome may be due to several reasons (i.e person, methods, environmental conditions, different CRM and chemicals and instruments used). Whereas, the combined uncertainty is the combination of all other factors. The budget of uncertainty comprises total uncertainties because of the earlier-mentioned elements (Cortez, 1995; Örnemark, 2004). Uncertainty is measured at about a 68 percent confidence interval. As far as ISO: 17025 is concerned, the testing laboratories essentially signify their uncertainties with distinct levels of confidence, which is known as the expanded uncertainty. Uncertainty (Nazir et al., 2020; Aslam et al., 2021; Van der Veen & Cox, 2021).

Combined uncertainty= √ $(U(x1))^2 + (U(x2))^2 + (U(x3))^2 + (U(x4))^2$

Expanded uncertainty = Combined uncertainty x level of confidence.

Robustness

The capability of any analysis method to remain unaffected by small changes in experimental conditions.

Method

The first humic acid sample was filtered, and then 5 5ml the filtrate in volumetric flask (100 ml volumetric flask). Added 50 ml of the extraction solution and shook for one hour through a mechanical shaker at 270 rpm. Made the volume of the extraction solution up to the mark. Centrifuged at 4000 RPM for 20 minutes to remove inert matter.

Analyst-1		
Sr. No.	**Repeat**	**HA=10%**
1	1	10.2
2	2	10.25
3	3	10.15
4	4	10.27
5	5	10.29
6	6	10.22
7	7	10.26
8	8	10.29
9	9	10.24
10	10	10.17
	Average%	10.234
	Stdev	0.0484
	RSD%	0.4725

Then added Nitric Acid (concentrated) in the filtrate till the ph drops to 1. Kept the sample for 2.0 hours to complete the reaction. Humic acid gets precipitated. Oven dried the filter paper (Whatman No. 42) till constant weight and recorded its weight. Collected the precipitates by filtration through Whatman No. 42. Dried the precipitates in the oven at

105 o C till constant weight. Finally recorded dry precipitates weight.

Calculations

Therefore, this method is suitable for achieving good quality and reliable results. Table 2: Repeatability findings of Humic Acid Fertilizer

Humic Acid (%) =

Where:

Weight of oven-dry precipitates x

100 Sample

(volumetaken)

Product Name	Company	Company Guaranteed Contents
Factor Plus	Suncrop Pesticides	Humic Acid: 10%

Weight of dry precipitates = weight of oven-dried precipitates along with filter paper - weight of oven-dried filter paper Table 1: Details of the Sample used in the study

Repeatability
The nearness of the agreement amongst the independent outcomes was got using the same protocol on same test matrix, under similar environments (similar analyst, similar equipment, and similar lab and within short interval of time) the measurement of repeatability is considered as relative standrd deviation qualified with the term: 'repeatability' as repeatability RSD.
Factor Plus Humic Acid (HA=10%) of Suncrop Pesticides was employed for repeatability, reproducibility, as well as earlier studies. The data of ten (10) replications (Table 2) predicts that the Humic Acid protocol is quite repeatable with the relative standard deviation (%RSD) of 0.4725 % as it is <10% representing homogeneousness of the obtained data. Henceforth the said parameter is considered as qualifies.

ReproducibTable 3
the data (Table 3) explain the nearness of agreement among Humic Acid results achieved independently with the same protocol over the same testing matrix, however, under dissimilar conditions (dissimilar scientist, dissimilar environment and afterward dissimilar interval of time). The T-test was used during this validation experiment.
Table 3: Humic Acid Reproducibility Results

S.No.	Analyst 1	Analyst 2
1	10.2	10.26
2	10.25	10.28
3	10.15	10.24
4	10.27	10.19
5	10.29	10.17
6	10.22	10.25
7	10.26	10.26
8	10.29	10.23
9	10.24	10.16
10	10.17	10.23
Average (X)	10.234	10.227
SD	0.0484	0.0406
Precision (%RSD)	0.473	0.397

t- test= =((10.234-10.227)/SQRT ((0.0484)×2/10)+(0.0406)×2 / 10) = 0.05
Tabulated -t =2.262 at 95% level of confidence
By the t-test, the calculated t -t-value (i.e 0.05) is less than the t-tabulated (i.e., 2.262); therefore, the results are statistically non- significant with each other, Therefore, the protocol is capable of delivering reproducible results, though duplicating

analysis with the standard deviations, i.e ± 0.0484 and ± 0.0406%, respectively, achieved by the two dissimilar scientists performing individually at dissimilar intervals of time. Reproducibility is supposed to be effective; henceforth, the parameter is qualified.

The %RSD of reproducible results was compared to the predicted relative standard deviation = PRSD(r). The PRSD(r) was calculated from the Horwitz formula: PRSD(R) = 2C -0.15

Where C is expressed as a mass fraction. The RSD(r) was found to be lower than the PRSDr, and hence the method was acceptable.

The Horwitz ratio or HorRat value

Horwitz ratio or HorRat value is a very simple performance parameter which reveals the acceptability of any analytical method regarding precision.

It is defined as the ratio of the Relative Standard Deviation of Reproducibility (RSDR), in percentage and is calculated from the reproducibility data, to the Predicted Relative Standard Deviation of Reproducibility (PRSDR) from the equation given by Horwitz, thus:

HorRat = RSDR/ PRSDR

The empirical acceptance range of HorRat is 0.5 to 2.

Table 4: Reproducibility of results of analysis of Humic acid by 2 analysts

Parameters	Analyst I	Analyst II
Relative Standard Deviation (Reproducibility) RSD_R	0.471	0.397
Predicted Relative Standard Deviation (Reproducibility) $PRSD_R$	2.83	
HorRat value	0.167	0.141

The Horwitz equation describes the relationship between the concentration of an analyte and the expected variability of the analytical method. The equation shows that:

The Horwitz equation is widely used in analytical chemistry to:

- Predict the expected variability of analytical methods
- Evaluate the performance of analytical methods
- Compare the performance of different analytical methods

Method Detection Limit (LOD)

By definition, the method limit of detection (LOD) is the lowest quantity of any ingredient which could be assessed as well as reported with 95% confidence level that the analyte concentration is > 0 and was calculated from the analysis of any material containing the particular analyte. The LOD of this study was 0.145 % Humic Acid in a given sample after multiplication by the method factor. 10 spiked samples of data were employed for determining the Limit of Detection.

LOD = blank value + k.s

Wherever:

k it is the factor that is multiplied with the standard deviations to calculate the uncertainty. Under prevailing situation, a factor (3) was used.

s= standard deviation for natural specimens without content, but for specimen having very low content or for the blank specimens. In this study, no any blank value was used as machine is already adjust to 0 (zero) for each reading. Subsequently, the standard deviation is for calculating the reproducibility of the laboratory. LOD = value of blank + $k.s_r$ = 0 + 3 × 0.0484= 0.145%

Estimation of Humic Acid Content in Fertilizer (H.A=10%)	Case # Ref:

Quantitation Limit of Method (LOQ)

S/N	Analyst 1	Analyst 2
1	10.2	10.26
2	10.25	10.28
3	10.15	10.24
4	10.27	10.19
5	10.29	10.17
6	10.22	10.25
7	10.26	10.26
8	10.29	10.23
9	10.24	10.16
10	10.17	10.23
Average	10.234	10.227
SU	0.0484	0.0406
Max SU:	0.0484	

The LOQ is the lowest concent
ration of any substance which could be determined with an ace ptable range. In practice, the LOQ is calculated by the best conventions to be t he analyte concentration.

Conforming to the obtained
standard deviation at a very low level multiplied by the factor, kq, which is normally used as ten
(10). The quantification limit obtained in this study was 0.484 % Humic Acid (in a given fertilizer) after multiplication by a factor. The LOQ in this situation is determined as being the value of blank plus
10 times the SD of the repeatability, as explained under:

UNCERTAINTY BUDGET								
S/ N	So urc es of Un cert ain ty	Unce rtaint y	Typ e A/B	K Fact or (Wh ere App lica ble)	Unce rtaint y Contr ibuti on	Average or Value	Rel ativ e Unc erta inty	Co mbi nin g Unc erta inty
1	An aly st	0.0484	A	1	0.0484	17.921	0.002700742	7.29401E-06
2	Vol . Fla sk 100 ml	0.01	B	2	0.005102041	99.77	5.1138E-05	2.6151E-09
3	Vol um etri c Fla sk 1000 ml	0.11	B	2	0.056122449	999.4	5.61561E-05	3.15351E-09
4	Pip ett 05 ml	0.002	B	2	0.001020408	4.98	0.000204901	4.19845E-08
5	Eq uip me nt (Ov en)	0.7	B	2	0.357142857	106	0.003369272	1.1352E-05
6	An alyt ical Bal anc e	0.00006	B	2	3.06122E-05	2	1.53061E-05	2.34277E-10
7	En vir on me nt	0.05	A	1	0.05	25.66	0.001948558	3.79688E-06
	Co mb ine d	0.0047	**@**	**95 % CL**				

LOQ = Blank + k.sr = 0 + 10 × 0.0484= 0.484%

Recovery

The recorded recovery of the Humic acid sample (i.e 10.234 %) is within the suggested limit of the standard criterion (i.e. ± 5%) of the recovery (Table 4), Therefore, the protocol under study is confirmed in this regard and is qualified.

Table 4: Evaluation of Humic Acid Recovery

S. No.	**Sta nda rd Mat rix**	**Detail of Sample**	**HA % Exp ecte d**	**HA% Obse rved**	**Recovery (%) (Obs/exp) ×100**	**Verificat ion range (± 5 % of 100% Recover y)**	**Co mm ents**
1	Hu mic Aci d sam ple	10% HA	10	10.234	102.34	95– 105 %	Veri fied

Bias

QUATEST3 (www.quatest3.com.vn)

Lab Name	Lab No.	Sampl e code	Lab Res ults	Expan ded Uncert	Z-Sc ore	Remar ks

				ainty		
SWTL, D.G.K han	Lab-05	QUAT EST3 QPT 029/24	9.95	0.024	0.7 6	Satisfa ctory

Uncertainty

	Un cert ain ty (Uc)			
	CL (K)	2	2	2
	Ex pa nd ed Un cert ain ty (Ue)	0.009 5	@	2
	Ex pa nd ed Un cert ain ty per uni t	0.000 1	%	

Summary

S. No.	Paramete r of Validati on	Limit / Range	Results	Comments
1	Referenc e Material	Humic Acid (10 %)	10.234	Qualifies
2	Repeatab ility	RSDRepeati bility <10 %	RSD=0.4 725%	Qualifies
3	Reprodu cibility	T-Calculate d < 2.262	Tcal = 0.05	Qualifies
4	Reprodu cibility	RSDReprod ucibility <10 %	RSD=0.4 725%	Qualifies
5	**Horwitz** ratio or HorRat value:	0.5-2.0	Within the admissibl e range	
6	Limit of Detection	< 5.0 Excellent <10 Acceptab le	0.145%	Qualifies
7	Limit of Quantific ation	< 10 Excellent <15 Acceptab le	0.484%	Qualifies
8	Recovery	95- 105 %	102.34%	Qualifies

Conclusion

The results of the validation study indicated that the Soil and Water Testing laboratory, Dera Ghazi Khan, is qualified to conduct Humic acid analysis using the proposed method by standards.
Method No. 973.04 chapter 2; 53.

Recommendations

This method will perform well in all the laboratories with all requisite machinery, NIST Traceable CRM and calibrated equipment with similar environmental conditions.

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