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Research Article

Validation and Development of Microkjeldahl Distillation Method for Amino Acid Determination

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ABSTRACT

To determine the amounts of nutrients they contain, fertilizer samples are tested; however, the results vary based on the techniques. Therefore, developing and validating a technique for Amino acid determination using Microkjeldahl Distillation Method was the main goal of this study. The Soil & Water Testing Laboratory (SWTL) Dera Ghazi Khan (accredited ISO/IEC, 17025:2017) approved the Microkjeldahl Distillation Method for measuring the amounts of Amino acid. The validation method included bias, recovery, limit of quantification, limit of detection, repeatability, and reproducibility and T-test was used for reproducibility. The limits for detection and quantification were, respectively, 0.74% and 2.46%. The reproducibility indicated T-calculated values of 0.862, which were lower than the T-tabulated (2.262), with repeatability RSD of 3.151%. The Amino acid recovery rate was 99.40%. Vietnam's findings (QUATEST3 www.quatest3.com.vn) had Z-scores that were within the acceptable range. The estimated values of Amino acid and their genuine values are strongly correlated, as indicated by recovery (i.e 99.40%). This outcome shows that the approach performed at its peak. Because all of the parameters worked well and produced accurate findings according to accepted standards. Therefore, the method might be used to determine the amount of Amino acid in fertilizers really well.

Keywords: Amino acid, Microkjeldahl, Distillation, Development, Validation.

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INTRODUCTION

Amino Acids are defined as “essential unit responsible for protein molecule formation”. Amino acids are organic compounds containing amine (NH_4) and carboxyl (COOH) group in addition to alkyl (R) group which are specific for each Amino Acid (AL-Modhafer, 2009). Amino Acids are bio activators which provide energy to the plants, lose during respiration and decomposition processes. Amino Acids have high melting point because they are hybrid ions. In plants amino Acids are found in two forms,1 freely and secondly in combined form for making proteins and peptides compounds.(Abd EL hafez,2011).Amino Acids are located in mitochondria and in chloroplast of the plants ,as result of the availability of kenotic acids (Beavers ,1991).The quantity of amino acid varies from plant to pant depending upon metabolic process. (Abed, 2007).Amino Acids are responsible for enhancing proteins productions, cell division ,natural hormones like GA3,IAA etc (Ahmed and El-Hameed,2003;Ahmed et al.,2007 and 2014).Studied showed that proline accumulation is mean for gathering nitrogen (Edress ,2009).Spray of amino acids on wheat plants limited nutrient deficiency because they are take up easily

and are used in a straight line for protein production.Glycine is main constituent for construction of chlorophyll in plants as it enhance chlorophyll concentration which result higher rate of photosynthesis (Kandi et al 2016).

Amino acids are used as foliar to various crops .Its quality has prime importance to stimulate the growth and development of different plants because it assimilated and mineralized for various physiological and structural development .In this consequence, the quality of amino acids can be enumerated through various analytical techniques like Microkjeldal distillation approach. Keeping in view of these facts, its quality must be checked to get the maximum yield .However, its validation is utmost important which would play vital role in its quality attributes However Validation is proceed for proving any procedure, process, equipment, material, activity or system performs as expected under given set of conditions and also give the required accuracy, precision, sensitivity, severity, etc. When extended to an analytical procedure, depending upon the application, it means that a method works reproducibly, when carried out by same or different persons, in same or different laboratories, using different reagents, different equipments, etc. In this article we discussed about the strategy and importance of validation of analytical methods.

The validation was not mentioned in the current Good Management Practices (cGMP's) of 1971, and precision and accuracy were confirmed as laboratory controls. The need for validation was disguised only in the cGMP instruction of March 1979. It was carried out in 2 sections: (one) Section 211.165, where the word 'validation 'was used and (second) section 211.194, in which the evidence of correctness, accuracy and reliability was made required for regulatory submissions.

The validation has following steps:

Accuracy, Precision (repeatability and reproducibility), Linearity and range, Limit of detection (LOD)/ limit of quantization (LOQ) limit of quantification, Selectivity/ specificity, Robustness/ ruggedness, Stability and system suitability studies.

The main benefit of analytical technique validation is that it Improve self-confidence level, for both developer as well as the consumer. Although validation practice is costly and time consuming, it results economical, eliminates annoying Repetitions and leads to better time running in the end.

MATERIALS AND METHODS

Repeatability

The relative standard deviation(SD), also recognized as "repeatability," is measure of extent of agreement between independent results that were obtained by using same analytical method on the same test material, under the identical circumstances (same user, apparatus, and laboratory) after a little time interval.

Accuracy

Usually talking, accuracy such the relative standard deviation Amino Acids were calculated based on repeatability and reproducibility. The repeatability test for amino acid was done in a laboratory using similar apparatus, personnel, and little time intervals. With the relative standard deviation, the repeatability measurement was done. The SD was done to compute the repeatability measurement.

Limits of Detection (LOD) and Quantification

The compassion of the Distillation unit was evaluated using LOD and LOQ computations. The smallest concentration of any substance that is merely clear and easily differentiated from zero, but not quantifiable, is called the lowest detectable dose (LOD). Gonzalez et al. (2010) and Renger et al. (2011) state that the lowest quantity of any substance that can be evaluated with a reasonable degree of precision and accuracy is referred to as the limit of quantification (LOQ).

Recovery

The accuracy of the system in examination was verified by the Amino acid revival computations. for assessment of accuracy , recoveries experiments were done to confirm amino acid loss due to contamination during sample preparation and matrix intervention through analysis. For analyte concentrations of 1g/mL, Taverniers et al. (2004) state that the desirable range of the recovery is 95% to 105%.The Eurachem Guide was used for uncertainty measurement. The operative, the analytical system, the accommodations and surrounds, the reagents and equipments, and other factors might all contribute to this uncertainty in the results. The collective effects of all the formerly listed components result in combined uncertainty. The budget for uncertainty included all components of uncertainty (Cortez, 1995; Örnemark, 2004). The judgment of uncertainty was performed

using a 68% confidence level. The testing laboratories must explain their uncertainties as extended uncertainty and give a clear confidence level in accordance with ISO/IEC 17025 standard (Aslam et al., 2021; Nazir et al., 2020; Van der Veen and Cox, 2021).

Total Nitrogen (Protein +Non Protein)

Liquid sample was taken. Filtered it through a Whatman filter paper No. 40. One ml filtered sample was taken in digestion tube .One gram of digestion mixture (K2SO4+CuSO4 :: 9:1) and ten to twelve ml of analytical grade of H2SO4 were in the digestion tube and this digestion tube was placed in digestion block and were heated upto 400C∘ in fume hood for two hours until the content in the tube turned from black to light green end point ..Now tube were detached from the digestion chamber and waited for cooling the content in the tubes. After cooling, the samples were ready for distillation process.

Distillation

Distillate suitable volume on the distillation Unit Velp UDK, fifty ml NaOH and 10ml distilled water were added in the digestion tube. Switched on the Velp UDK, heated and collected distillate in 4% boric acid receiver flask (40 ml 4% Boric Acid was taken), 3 to 4 drops of BCG (Bromocresol Green) were added. Purple color was developed and was changed to golden yellow on distillation. Stoped the Unit Velp UDK, removed receiver flask, titrated it against 0.1 N H_2SO_4 from golden yellow to a purple end point. Noted the reading of sulfuric acid used.

Calculations

$$\%N = \frac{14.1 \times \text{ml of titrate for sample - ml of titrate for blank} \times \text{N of acid} \times \text{d.f.}}{\text{Weight of sample (g)} \times 10}$$

(Jones, Jr. J. B. 1991) and also (AOAC Official Method 892.10) Fertizer chapter 2 Page 15.

Non Protein Portion

One ml filtered sample was taken in centrifuge tube, also ten ml ice cold TCA solution (50%) were added, centrifuge for fifteen minutes @ 4000 RPM was done .Protein were precipitated .washed precipitate with TCA ice cold and filtered .All supernatants were taken in 25 ml volumetric flask and remaining volume were added upto mark with TCA solution. Ten ml aliquot sample was taken in digestion tube, 10-12 ml analytical grade H_2SO_4 were added, digestion were done at four hundred centigrade for two hours. End point was bluish green. Distillate suitable volume on the distillation Unit Velp UDK, fifty ml NaOH and 10ml distilled water were added in the digestion tube. Switched on the Velp UDK, heat and Save distillate in 4% boric acid receiver flask (40 ml 4% Boric Acid was taken), 3 to 4 drops of BCG (Bromo Cresol Green) were added. Purple color will form and will be changed to golden yellow on distillation. Stop the Unit Velp UDK, remove receiver flask, titrated it against 0.1 N H_2SO_4 from golden yellow to a purple end point. Note the reading of sulphuric acid used.

Formula for calculating the Non-Protein Portion.

$$N\% = \frac{0.00141 \times 100 \times 25}{\text{Sample taken (10 ml)}}$$

After Using the formula the factor was 0.3525.

RESULTS

Repeatability

The proximity of the agreement among independent outcomes was obtained using the same protocol on the identical test matrix, under comparable conditions (similar analyst, similar equipment, and a consistent laboratory environment within a short time frame). The measurement of repeatability is considered as the relative standard deviation, designated as "repeatability" RSD. Isabion amino acid (A.A 10%) of syngenta group was analyzed for repeatability, reproducibility as well as earlier studies.

The data of ten (10) replications (Table 1) indicates that the Amino Acid protocol is quite repeatable with the relative standard deviation (%RSD) of 3.151% as it is <10% representing homogeneous of the obtained data. Hereafter the said parameter is considered as qualifies.

Table.1 Repeatability for analysis results of Amino acid

Repeat	Amino acid=10%
1	9.88
2	10.13
3	9.94
4	10.31
5	10.75
6	9.62
7	9.75
8	10.00
9	9.97
10	9.93
Average	10.03
Standard Deviation	0.316
RSD%	3.151

Table. 2. Reproducibility results for analysis results of Amino acid

Repeat	Analyst-1	Analyst-2
	Amino acid (%)	Amino acid (%)
1	9.88	9.79
2	10.13	10.10
3	9.94	9.86
4	10.31	9.93
5	10.75	10.01
6	9.62	9.82
7	9.75	9.87
8	10.00	10.15
9	9.97	9.95
10	9.93	9.88
Average	10.03	9.94
Standard Deviation	0.32	0.12
Relative Standard Deviation	3.151	1.193

T-Test
T-calculated = (10.03-9.94)/SQRT((0.32*0.32/10)+(0.12*0.12/10))
t- Calculated = 0.862
t- Tabulated = 2.262

The data (Table 2) demonstrate the consistency of agreement among amino acid results obtained independently using the same protocol on the same testing matrix, despite being conducted under differing conditions (different scientist, different environment, and subsequent varying intervals of time). The T-test was used during this validation experiment.

Based on the t-test, the computed t-value (i.e., 0.862) is less than the critical t-value from the table (i.e., 2.262); therefore, the results are not statistically significant from each other. Therefore, the method is passed to produce the reproducible results under dissimilar conditions with the standard deviations, i.e ± 0.32 and ± 0.12%, respectively, achieved by the two dissimilar scientists performing individually at different intervals of time. Reproducibility is supposed to be effective; henceforth, the parameter is qualified.

The LOD of this study was 0.74% Amino Acid in a given sample after multiplication by the method factor. 10 spiked samples of data were employed for determining the Limit of Detection.

While the quantification limit in this study was 2.46% amino acid in given sample after completion the multiplying factors. The LOQ in this analysis was determined as being the value of blank plus 10 times the SD of the repeatability.

Table.3 Limit of Detection (LOD) and Limit of Quantification (LOQ) results for analysis results of Amino acid

Blank+ spike (AA=10%)	Repeat	Sample reading			Method LOD %	Method LOQ %
Batch		Amino acid %	SO	SO'		
	1	9.88	0.110	0.447	0.74	2.46
	2	10.13				
	3	9.94				
	4	10.31				
	5	10.75				
	6	9.62				
	7	9.75				
	8	10.00				
	9	9.97				
	10	9.93				
Standard Deviation	so	0.32	so = STDEV	so' =SQR(2)*so	LOD=3*SO'	LOQ=10*SO'

Slope= sqrt(2)*Standard Deviation
LOD **=** 3*Standard Deviation/Slope
LOQ **=** 10*Standard Deviation/Slope

Table.4 Evaluation of Recovery of analysis results of Amino acid

S. No.	Standard sample	Sample detail	Amino acid (%) Expected	Amino acid (%) Observed	Recovery (%)	Verification range (± 20 % of 100% Recovery)	Remarks
1	Amino acid (Isabion) Syngenta	Amino acid (10% W/V) (Isabion) Syngenta	10.0	9.94	99.40	80 - 120 %	Verified

DISCUSSION

The consistency and precision of systematic techniques are fundamental in evaluating biochemical ingredients for example amino acids, predominantly when these are applied as bio-stimulants or nutrient supplements in agronomic practices. The current assessment focused on key validation parameters comprising repeatability, reproducibility, limit of detection (LOD), limit of quantification (LOQ), and recovery—each serving as a critical indicator of the method's analytical robustness and suitability for agronomic research applications.

Repeatability

The repeatability results revealed tremendous consistency, with amino acid determinations ranging from 9.62% to 10.75% (average = 10.03%) and a relative standard deviation (RSD) of 3.15%. This assessment is well within the standard range (<5%) suggested for chemical and biochemical analyses (Lucini et al., 2020). The narrow deviation among replicate measurements indicates high stability of the analytical method and minimal instrumental or procedural variability. Such precision confirms consistency in routine laboratory estimations of amino acid formulations used for crop bio-stimulation and foliar nutrient management (Popko et al., 2018).

Reproducibility

The inter-analyst reproducibility assessment supplementary reinforced the method's credibility. The calculated t-value (0.862) was lesser than the tabulated value (2.262), endorsing that no statistically significant difference occurred between the results of the two analysts. The RSD values (3.151% and 1.193% for Analyst 1 and Analyst 2, respectively) indicate satisfactory analytical harmony, reflecting upright training uniformity and method standardization within the laboratory environment. According to Motsara and Roy (2008), reproducibility within

laboratories is a key benchmark for analytical credibility, particularly when data are intended for agronomic formulation registration or quality control.

Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD (0.74%) and LOQ (2.46%) values derived from the standard deviation and slope of calibration data fall within acceptable analytical sensitivity limits. These low thresholds indicate that even minor concentrations of amino acids can be detected and quantified with confidence. Such sensitivity is essential for agronomic formulations where amino acids are present in complex matrices or when assessing the residual levels post-application in soils and plants. The method's capacity to reliably quantify small variations aligns with current analytical quality standards for biofertilizer and biostimulant evaluation (Povero et al., 2016; FAO, 2024).

Recovery

The recovery rate of 99.40% sanctions the precision of the analytical method, as it falls within the satisfactory range of 80–120% suggested for agricultural product validation (AOAC, 2023). This result shows that the technique neither overestimates nor underestimates amino acid concentrations, thus authenticating its efficacy in measuring amino acid-based products such as Isabion (Syngenta). High recovery precision guarantees data reliability in both study and commercial quality assurance contexts. Alike validation patterns were described by Williams et al. (2023), highlighting that near-complete recoveries reflect optimum extraction efficiency and minimal matrix interference.

CONCLUSION AND RECOMMENDATIONS

In summary, the analytical authentication of the amino acid determination method demonstrated creditable precision, reproducibility, sensitivity, and exactness. The close agreement among repeated measurements and the lack of any significant difference between analysts reveal the method's high level of reliability. The calculated LOD and LOQ sanction that the method is accomplished of noticing understated disparities in amino acid concentration—an essential feature when analyzing multifaceted formulations used in field applications. The recovery performance, approaching full precision, sanctions that the analytical procedure can quantify amino acid content without interference from other formulation components. This fortifies its applicability for the quality control of biostimulants and amino acid-based nutrient products used in agronomy. Together, these validation results show that the method is vigorous and appropriate for purpose, meeting the analytical standards outlined by AOAC (2023) and ISO 17025. By ensuring dependable results, this technique supports the development and evaluation of amino acid-based biostimulants aimed at improving plant nutrition, stress tolerance, and overall productivity. Reliable analytical data serve as a cornerstone for evidence-based agronomy, linking laboratory precision with field-level performance. As a result, the authenticated method can be employed definitely for routine lab analysis, product formulation verification, and future agronomic investigation concentrating on nutrient bioavailability and crop response effectiveness. This method can be safely used with high precision and accuracy if applied as such as described in Material and Method section.

AUTHOR CONTRIBUTIONS

Rehamat Ullah and Muhammad Akram Qazi conceptualized and designed the study. Fareeha Habib, Farhat Bashir, Sobia Noor, and Abid Niaz conducted the experiments and collected the data. Muhammad Bilal and Amir Afzal compiled, analyzed, and prepared the final manuscript based on the research findings. All authors contributed substantially, worked collaboratively, and approved the final version of the manuscript, ensuring the successful completion of the project.

COMPETING OF INTEREST

The authors declare no competing interests.

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