Analysis of Total Amino Acids in Rice Using a Validated Reversed-Phase High Performance Liquid Chromatographic Method with Diode Array Detection (RP-HPLC-DAD)

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Abstract

Adaptable to any routine laboratory, the study presents the validation results of a simple, accurate and reliable method developed for the analysis of total amino acids (TAAs) in rice using reversed-phase high-performance liquid chromatography-diode array detection (RP-HPLC-DAD).

Exhibiting excellent selectivity with resolution (R_s) ≥ 2 for seventeen amino acids, the method was proven accurate against the analysis performed on the certified reference material (CRM): NIST 3233. Percentages of recoveries were in the range 86%-100% with percentage relative standard deviation (%RSD) $\leq 6\%$ for all amino acids. Limit of detection (LOD) and limit of quantification (LOQ) values were within 0.024-0.069 g/100 g and 0.025-0.078 g/100 g respectively. A wide working range with satisfactory linearity having regression coefficients ≥ 0.999 were reported for all the amino acids.

Complying with international guideline requirements, this validated method can be successfully applied for the determination of seventeen TAAs including all essential amino acids in rice.

Keywords

Rice, RP-HPLC- DAD, Total amino acids, Validation

Introduction

As the dietary staple of more than half of the world population [1], rice (*Oryza sativa* L.) is recognized as one of the most important cereal crops in the world. Rice significantly contributes to the daily nutritional requirement as the major source of en-

ergy supply and the protein intake of the Asian diet [2]. Rice contains an average protein content of 4.5-15.9% [2]. Owing to the changes in the cultivar, environmental conditions, breeding techniques, agricultural practices and postharvest conditions, significant variations in protein levels both quali-

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tatively and quantitatively were observed in rice [3,4]. Protein is the second most abundant nutrient present in rice which is only next to carbohydrates. Protein plays a significant role in determining the nutritional quality, functional properties, texture, pasting capacity and the sensory characteristics of rice [5-7]. The determination of amino acid composition of rice becomes vital for defining the characteristics related to the protein quality of rice. Several methods for determination of amino acids in rice and other matrices have been discussed in literature [8-14]. In amino acid analysis, generally proteins are hydrolyzed using 6 mol/L HCl solution at the temperature of 110 °C [8,9,13,15] for extended hours of 24 h or at temperatures above 110 °C for periods shorter than 24 h [9,16,11]. The hydrolyzed amino acids are then analyzed using either high performance liquid chromatography with ultra violet/visible, diode array or fluorescence detection (HPLC-UV/VIS/DAD/FLD) [10-12,16], amino acid analyzers [13,17], liquid chromatography-tandem mass spectrometric detection (LC-MS/MS) [14,18,19] gas chromatography-mass spectrometric detection (GC-MS) [14,19,20] or near-infrared reflectance spectroscopy (NIRS) [21].

It has been reported that the standard hydrolysis conditions do not guarantee the extraction of all the amino acids, most often excluding sulfur containing amino acids and tryptophan [15]. The studies have further indicated that amino acids analyzed under standard hydrolysis conditions can lead to either underestimation or over estimation of the actual value affecting the reliability of the results produced [15,22]. For example, losses encountered in tryptophan during the acidic hydrolysis can be overcome by performing the alkaline hydrolysis during the tryptophan analysis [10,23]. Thus the need for accurate and reliable methods to determine amino acid composition in rice becomes extremely important in defining its protein characteristics. The characterization of optimum hydrolysis conditions that produce acceptable recoveries for individual amino acids in the matrix assures the generation of reliable results. Therefore, evaluation of the method performance characteristics becomes paramount for the generation of accurate results.

There are several studies carried out on analysis of amino acids in rice [13,17,24-26] or other cereals [27-29]. However, except for the study on pseu-

do-cereals by Mota, et al. 2016 [11] and work by Szkudzinska, et al. 2017 [30] on rye, none of the work described in recent studies outlines a comprehensive validation study aimed at the evaluation of the method performance characteristics in analyzing the profile of amino acids in rice or cereal matrices which includes the complete profile of essential amino acids. The method developed by Mota, et al. 2016 [11] has excluded tryptophan analysis, hence the complete essential amino acid composition of the cereal matrix was not covered. Further, in their work, the evaluation of method performance characteristics has been performed based on the analysis of an ephedra containing protein powder which is a non-cereal, hence challenges the validity of the performance evaluation criteria. On the other hand, as done by Szkudzinska, et al. 2017 [30], performance of the method evaluated based on fortification of amino acids in free form does not guarantee the actual recoveries that would be obtainable for the protein bound amino acids. Most often sulfur containing amino acid analysis, involves an oxidation step of 16 h prior to hydrolysis [8,9,30].

Primarily, due to these complexities involved with the hydrolysis and the unavailability of a simple and reliable protein hydrolysis method, amino acid analysis often remains unattempted by most of the analytical laboratories in the routine framework. In this context, the aim of this study is to develop and validate a simple, accurate and reliable method that enables analysis of amino acid composition including all essential amino acids in rice with the use of the conventional laboratory oven and the RP-HPLC-DAD detection making the analysis simple and accessible to any routine analytical laboratory.

Methodology

Materials and methods

Chemicals and reagents: The standard reference materials of amino acids; L-aspartic acid (Asp), L-serine (Ser), L-glutamic acid (Glu), L-lysine (Lys), L-proline (Pro) glycine (Gly), L-histidine (His), L-arginine (Arg), L-threonine (Thr), L-alanine (Ala), L-tyrosine (Tyr), L-valine (Val), L-methionine (Met), L-isoleucine (Ile), L-leucine (Leu) and L-phenylalanine (Phe) (Sigma Alrich, Chemicals, St. Louis, MO), each of purity > 98% were prepared in 0.1 M HCl solution. Due to the limited stability in acidic solutions, L-tryptophan (Trp), L-theanine and L-norvaline

(Nva) were prepared in ultra pure water and were stored for only two weeks. The certified reference material (CRM): NIST 3233 which was a fortified breakfast cereal was purchased from the National Institute of Standards and Technology (NIST), USA. L-Theanine (Baxter Smith Labs, USA), and L-Norvaline (Sigma Alrich, Chemicals, St. Louis, MO) with purity > 98% were used as the internal standards (ISs).

The derivatization of the primary amino acids was carried out using the o-phthalaldehyde 3-mercaptopropionic acid (OPA-MPA) as the derivatizing agent, while the secondary amino acid: Pro was derivatized using the 9-Fluorenylmethoxycarbonyl chloride (FMOC) (Agilent Technologies, USA). Other chemicals which were of analytical reagent grade and the HPLC grade solvents purchased from Sigma Aldrich were used for the preparation of samples and the two mobile phases which consisted of A(40 mmol/L Na₂HPO₄ with pH adjusted to 7.8 using a 10 mol/L sodium hydroxide solution) and B (45% acetonitrile, 45% methanol, 10% water).

Grain samples: The applicability of the method was assessed [31–33] using seven traditional rice varieties obtained from the regional rice research and development centers (RRRDCs) located at Batlagoda and Bombuwala in Sri Lanka.

Sample preparation

The unpolished rice samples after finely grinding using a laboratory grinder (IKA-MF 10 basic Micro-fine grinder drive), were sieved through a 0.3 mm sieve prior to analysis. To 0.2 g of the sieved sample placed inside a screw capped glass tube, 5.00 mL of the hydrolysis mixture (6 mol/L HCl containing 1% (v/v) thiodiglycol and 1 g of phenol per liter) was added [8] and vortexed for 5 minutes. The vortexed sample was placed inside a drying oven set at 110 °C. During the first hour, in order to prevent a building up of pressure (due to the evolution of gaseous substances) and to avoid explosion, the screw cap was placed over the top of the glass tube without tightening. After 1 h, the glass tube was closed and left in the oven for 22 h. On completion of the hydrolysis, the glass tube was removed from the oven and once the mixture reaches the room temperature was carefully opened inside an ice-water bath. Initially, the pH of the hydrolyzed mixture was adjusted to around pH to 3 using a 10 mol/L sodium hydroxide solution while making sure that the

temperature of the solution was kept below 40 °C. The final adjustment of pH to 2.2 was carried out using a 1 mol/L sodium hydroxide solution. Finally the pH adjusted solution was transferred to a 25 mL volumetric flask and after addition of 100 µL of 50 nmol/L of both IS's L-Nva and L-theanine, the resulting solution was made up to the mark with ultra pure water acidified to pH 2.2 with a 0.01 mol/L HCl solution.

Since Trp is destroyed during the acid hydrolysis, alkaline hydrolysis was performed using a 4.2 mol/L NaOH solution containing 1% (v/v) thiodiglycol for 18 h instead of the acid hydrolysis mixture mentioned above. The alkaline mixture resulting from the hydrolysis was adjusted to pH 2.2 with the use of 6 mol/L HCl solution finally being made up to 25 mL with ultra pure water acidified to pH 2.2 with a 0.01 mol/L HCl solution.

The prepared samples, after filtering through 0.45 µm syringe filter, were injected to the HPLC using automated pre-column derivatization.

HPLC analysis

The analysis was performed using an Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA) which consisted of a diode array detector (DAD) (G1321A). Pre-column online derivatization was achieved using the Agilent programmable auto sampler (G1313A). An Agilent Zorbax Eclipse AAA column with dimensions of (4.6 mm × 150 mm, 5 µm) was used for the chromatographic separation. The gradient elution started with 100% A for 1.9 min; ramped to 57% B within next 18.1 min; ramped to 100% B in 18.6 min and kept at 100% B till 22.3 min; then ramped to 100% A in 23.2 min and kept with 100% A till 26 min. The column was operated at 40 °C and the flow rate of the method was set at 2 mL/min throughout the runtime [34]. The Zorbax AAA guard columns (4.6 mm × 12.5 mm) were used to prolong the duration of the analytical column.

With the aid of the programmable autosampler and the injector program outlined in Table 1, the automated pre-column online derivatization with OPA-MPA and FMOC was performed prior to the injection of samples to the HPLC [34]. The derivatized primary amino acids were monitored at 338 nm while the secondary amino acid: Pro was monitored at 262 nm using the DAD detector. Agilent Chemstation software version B.04.03 was used for data acquisition and analysis.

Table 1: Injector program.

Line	Function	Amount	Vial position	Reagent
1	Draw	2.5 µL	1	borate buffer
2	Draw	0.5µL	7 onwards*	sample
3	Mix	3.0 in air max speed 2 times		
4	Wait	0.5 min		
5	Draw	0 µL	2	Water - (needle wash using uncapped vial)
6	Draw	0.5 µL	3	OPA
7	Mix	3.5 µL in air Max speed 6 times		
8	Draw	0.5 µL	4	Fmoc
9	Mix	4.0 µL in air Max speed 6 times		
10	Draw	0 µL	2	Water- (needle wash using water in uncapped vial)
11	Draw		6	acetonitrile
12	Draw	32 µL	5	water (capped vial)
13	Mix	18 µL in air max speed 2 times		
14	Inject			

*vial position of the sample in the tray.

Validation of the method

Validation method was carried out in compliance with the requirements specified in the international method validation guidelines [31-33]. The performance characteristics of the method in terms of accuracy, precision, recovery, selectivity, linearity, limit of detection (LOD), and limit of quantification (LOQ) were studied and the applicability of the method was tested on seven local traditional rice varieties.

The accuracy, precision, recovery and linearity of the method were evaluated based on the results obtained after the analysis of the certified reference material (CRM): NIST 3233 on breakfast cereals in six replicates.

In the absence of a matrix material free of amino acids, the determination of LOD and LOQ val-

ues were performed using six replicate analysis performed on the blank samples fortified at lowest quantifiable limits.

Statistical analysis

The statistical analysis was performed using the statistical software package, SAS for Windows V 9.1 (SAS Institute Inc., NC, USA). The level of significance was $p < 0.05$. Based on the percentage relative standard deviations (%RSD), intra and inter day precision and recovery values were evaluated. The estimation of the expanded uncertainty was evaluated at 95% confidence level including the factors contributing from repeatability, reproducibility and regression. The TAAs observed for the rice cultivars reported at the two locations were statistically analyzed using ANOVA and Duncan multiple range test.

Table 2: Accuracy of amino acid analysis.

Amino acid	Asp	Glu	Ser	His	Gly	Thr	Arg	Ala	Tyr	Val	Met	Trp	Phe	Ise	Leu	Lys
A_c (m/m%)	0.438	2.25	0.375	0.162	0.342	0.241	0.322	0.323	0.231	0.343	0.139	0.092	0.373	0.270	0.550	0.103
$ \bar{X} - A_c $	0.026	0.058	0.02	0.01	0.014	0.008	0.032	0.019	0.031	0.030	0.018	0.002	0.016	0.013	0.040	0.015
$2\sqrt{\mu_B^2 + \mu_D^2}$	0.042	0.172	0.051	0.027	0.027	0.014	0.054	0.034	0.046	0.030	0.018	0.028	0.028	0.019	0.040	0.036
s	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes

A_c : Assigned value of the certified reference material (CRM); $\bar{X}$: Mean value obtained for the CRM; μ_B^2 : Uncertainty associated with the certified reference value; μ_D^2 : Uncertainty associated with the analytical method for the particular analyte; *Acceptance criteria*: $|\bar{X} - A_c| \leq 2\sqrt{\mu_B^2 + \mu_D^2}$; Asp: Aspartic acid; Glu: Glutamic acid; Ser: Serine; His: Histidine; Gly: Glycine; Thr: Threonine; Arg: Arginine; Ala: Alanine; Tyr: Tyrosine; Val: Valine; Met: Methionine; Trp: Tryptophan; Phe: Phenylalanine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine

Results and Discussion

The summary of the validation data in terms of accuracy, selectivity, precision, recovery, limit of detection (LOD), limit of determination (LOQ), linearity and measurement uncertainty obtained for the studied amino acids are listed in Table 2, Table 3 and Table 4.

Accuracy

The accuracy was evaluated based on the recovery values obtained for the certified reference material (CRM): NIST 3233 on breakfast cereals. The acceptance criterion for accuracy was calculated as per the equation 1 where A_c : Assigned value of the certified reference material (CRM), $\bar{X}$: Mean value obtained for the CRM, μ_B^2 : Uncertainty associated with the certified reference value and μ_D^2 : Uncertainty associated with the analytical method for the particular analyte respectively. As summarized in Table 2, based on the criterion [35], the method was found accurate for analysis of all the studied amino acids.

Since an assigned values for Pro was not available in the CRM, accuracy of the analysis was assessed based on the recovery values obtained for the fortified Pro at the middle of the working range.

$$\text{Acceptance criterion: } |\bar{X} - A_c| \leq 2\sqrt{\mu_B^2 + \mu_D^2} \quad (1)$$

Recovery

Recoveries of the amino acids were calculated based on the assigned values mentioned for the certified reference material (CRM): NIST 3233 on breakfast cereals. Since a value was not assigned for Pro in the CRM, recovery of Pro was calculated based on the fortification of Pro to the rice matrix at the mid level of the working range.

During the acid hydrolysis, Trp is entirely destroyed while Gln and Asn are completely oxidized to Glu and Asp respectively. However, inclusion of 1% (w/v) phenol as a protective agent in the hydrolysis mixture significantly reduced the loss of recoveries of the sensitive amino acids such as Met, Ser, Thr and Tyr [9]. Base hydrolysis aided optimum recovery for Trp which is 98%. Therefore, the overall mean recoveries obtained for the studied amino acids as given in the Table 3 were in the range 86%-100%. Except for Tyr, Val, Lys, Pro, Arg and Met, the recoveries for rest of the amino acids were with-

Table 3: Precision, recovery and selectivity.

Amino acid	Precision (% RSD) (n = 6)		% Recovery (n = 6)	Selectivity	
	Intra - day	Inter - day		Difference in relative RT between spiked and blank samples (min)	Resolution (R _s)
Asp	2	3	94 ± 3	0.0010	17
Glu	2	2	98 ± 2	0.0012	24
Ser	2	4	100 ± 4	0.0008	10
His	3	5	100 ± 5	0.0005	4
Gly	2	2	94 ± 2	0.0007	2
Thr	3	4	99 ± 4	0.0008	5
Arg	3	4	90 ± 4	0.0004	9
Ala	2	4	96 ± 4	0.0003	13
Tyr	2	3	88 ± 3	0.0006	17
Val	3	6	90 ± 6	0.0013	4
Met	5	6	86 ± 6	0.0001	2
Trp	3	5	98 ± 5	0.0007	9
Phe	2	5	94 ± 5	0.0007	5
Ile	2	3	95 ± 3	0.0005	2
Leu	2	5	95 ± 5	0.0004	7
Lys	2	6	90 ± 6	0.0002	4
Pro	3	6	90 ± 6	0.0001	25

RSD: Relative Standard Deviation; RT: Retention time; n: Number of replicates; ±: Standard deviation of recoveries are given followed by the ± symbol in the respective cells; Asp: Aspartic acid; Glu: Glutamic acid; Ser: Serine; His: Histidine; Gly: Glycine; Thr: Threonine; Arg: Arginine; Ala: Alanine; Tyr: Tyrosine; Val: Valine; Met: Methionine; Trp: Tryptophan; Phe: Phenylalanine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Pro: Proline

in the accepted values for recovery for the specified analyte concentration levels recommended by the FDA Guidelines for the validations of Chemical Methods for the Foods Program [31].

Selectivity

The selectivity was assured based on the relative retention times calculated with reference to the respective IS obtained for amino acids in the sample to those obtained for amino acid reference standards. For polar amino acids, theanine was considered as the IS while non polar amino acids were normalized against the IS: Norvaline.

A minimal difference of ± 0.1% of relative retention times obtained for the amino acids in spiked matrices against the reference standards injected in blank solutions were considered acceptable. The resolution factors (Rs) calculated for all the amino

acids were greater than 1 with the lowest Rs value of 2 obtained for Gly/Thr and Phe/Ile pairs (Table 3), where generally Rs ≥ 2 is regarded adequate [36]. Signifying excellent resolution which indicates better selectivity for all the analytes of interest as given in the Figure 1.

Precision

The precision of the method was measured under repeatable conditions on six replicate analyses of the CRM on the same day while the intermediate precision was calculated based on six replicate analysis carried out on the CRM sample by different analysts on different days over an extended period of three months.

The intermediate precision in analysis of each amino acid in the method (%RSD) was ≤ 6% (Table 4). These RSD values are well in compliance with

Figure 1: Amino acid profile of: a) Standards mixture; b) Rice sample.

ASP: Aspartic acid; GLU: Glutamic acid; SER: Serine; HIS: Histidine; GLY: Glycine; THR: Threonine; ARG: Arginine; ALA: Alanine; TYR: Tyrosine; VAL: Valine; MET: Methionine; TRP: Tryptophan; PHE: Phenylalanine; ILE: Isoleucine; LEU: Leucine; NVA: Norvaline; LYS: Lysine; PRO: Proline; IS: Internal standard.

the recommended RSD for the particular analytical range which is 6% as specified in the AOAC guideline on Method validation [33].

Limit of detection and limit of quantification

The LODs and the LOQs were calculated considering the mean and the standard deviation (SD) of the values obtained for the analyzed blank samples fortified at the lowest detection levels. The values were calculated by adding to the mean value, 3 times of the SD for LOD while 5 times of the SD values for LOQ respectively [32]. The LODs and the LOQs for the method were in the range 0.024-0.069 g/100 g and 0.025-0.078 g/100 g respectively allowing high sensitivity in detection of amino acids in lower levels as given in Table 4. The method reported lowest and highest LODs and LOQs for Gly and Tyr respectively. Miniaturization of the final total volume of the neutralized sample after hydrolysis aided achieving of low LOD and LOQ values allowing quantification of amino acids in rice and other cereals present in low levels.

Linearity and the working range

The calibration range consisted of seven calibra-

tion levels at 25, 50, 100, 250, 500, 1000 and 2000 $\mu\text{mol/L}$. The linear regression line was constructed using the peak area ratio of standard to IS at each concentration level. The regression coefficients obtained for all amino acids analyzed (R^2) were ≥ 0.999 . Hence, excellent linearity is demonstrated in the method over a wide working range as given in the Table 4. This enables the analysis of amino acids levels which are generally present in rice and other cereals in different levels in a single run.

Measurement uncertainty

The measurement uncertainty for each amino acid was calculated by considering the uncertainty contribution arising from repeatability, regression, standard preparation and sample preparation of the method [37].

The percentage expanded uncertainties for each amino acid, with a coverage factor of 2 ($k = 2$) were $\leq 7\%$ for all the amino acids analyzed as given in Table 4. Except for Val and Met, Szkudzińska, et al. 2017 [30] reports uncertainties of similar magnitude in their method on amino acid analysis.

The uncertainties associated with the repeat-

Table 4: Working range, Linearity, LOD, LOQ and Uncertainty of the method.

Amino Acid	Working Range (g/100 g)	Regression Equation	R ²	LOD (g/100 g)	LOQ (g/100 g)	% Expanded Uncertainty (k = 2)
Asp	0.045-3.32	$y = 1.0164E + 06x - 3.1558E-01$	0.999	0.043	0.045	4
Glu	0.054-3.68	$y = 1.3315E + 04x + 1.5022E-02$	0.999	0.051	0.054	5
Ser	0.035-2.62	$y = 1.0467E + 06x - 4.5370E-01$	0.999	0.034	0.035	5
His	0.050-3.88	$y = 6.0263E + 05x - 1.6916E-01$	0.999	0.049	0.050	3
Gly	0.025 - 1.88	$y = 1.0718E + 06x - 4.6236E-01$	0.999	0.024	0.025	4
Thr	0.039 - 2.98	$y = 1.0313E + 06x - 2.7667E-01$	0.999	0.038	0.039	4
Arg	0.057 - 4.35	$y = 1.0328E + 06x - 5.3172E-01$	0.999	0.056	0.057	5
Ala	0.031 - 2.22	$y = 6.6305E + 06x + 2.5812E + 00$	0.999	0.030	0.031	3
Tyr	0.078 - 4.52	$y = 9.9886E + 05x - 4.4620E-01$	0.999	0.069	0.078	4
Val	0.046 - 2.92	$y = 1.2085E + 06x - 2.6690E-01$	0.999	0.042	0.046	7
Met	0.052 - 3.72	$y = 1.0892E + 06x - 4.5200E-01$	0.999	0.050	0.052	7
Trp	0.071 - 5.10	$y = 1.9063E + 07x - 9.8928E-01$	0.999	0.069	0.071	3
Phe	0.056 - 4.12	$y = 1.0562E + 06x + 1.3355E-01$	0.999	0.054	0.056	3
Ile	0.043 - 3.28	$y = 1.0258E + 06x - 2.0146E-01$	0.999	0.042	0.043	3
Leu	0.043 - 3.28	$y = 1.0717E + 06x - 1.7994E-01$	0.999	0.042	0.043	3
Lys	0.050 - 3.65	$y = 1.5634E + 06x + 2.5580E + 00$	0.999	0.048	0.050	5
Pro	0.043 - 2.88	$y = 1.2774E + 06x - 7.3935E-01$	0.999	0.040	0.043	4

LOD: Limit of Detection; LOQ: Limit of Quantification; R²: Regression Coefficient Coverage factor (k) = 2; Asp: Aspartic acid; Glu: Glutamic acid; Ser: Serine; His: Histidine; Gly: Glycine; Thr: Threonine; Arg: Arginine; Ala: Alanine; Tyr: Tyrosine; Val: Valine; Met: Methionine; Trp: Tryptophan; Phe: Phenylalanine; Ile: Isoleucine; Leu: Leucine; Lys: Lysine; Pro: Proline

ability of the method and the regression analysis involved in the calibration step mainly constituted the percentage uncertainty, while the uncertainty associated with the preparation of the calibration standards and preparation of samples made up the remainder.

Amino acid composition in rice samples

The Table 5 and Table 6 summarize the composition of TAAs determined using the validated method in seven rice varieties cultivated in two locations in the country. As per the composition, Glu was the major amino acid present while Asp, Ala, Val, Gly and Leu levels also found in comparatively higher levels. Further, compared to the other analyzed amino acids, Trp, Met and His were found in relatively lesser quantities. In literature, several studies report similar compositions of Glu, Asp and Val in rice [13,17,24,25]. However, in comparison to those studies, relatively higher levels of Ala and Gly have been reported in the local traditional va-

rieties.

When these data were statistically investigated using the Duncan Multiple Range Test it was revealed that there were significant variations ($p < 0.05$) in the individual amino acids and the mean total amino acid levels among the cultivars as well as between the two locations. The results were further analyzed to evaluate the impact of cultivar and location on the individual and total amino acid levels. Based on the analysis, it was revealed that there were significant variations in Asn, Glu, Thr, Arg, Ala, Tyr, Phe, Ile, Leu, Lys, Pro as well as mean total amino acid levels among the studied cultivars. Except for Gly, Thr, Arg, Ala, Phe and Ile, rest of the amino acids significantly varied between the two locations. Except for His, Tyr, Met and Pro, the interaction between cultivar (c) and the location (l), (c × l) was found significant for all the investigated amino acids, emphasizing the significance of impact of variety and the geographical conditions on

Table 5: Total amino acid contents of traditional rice cultivars grown in Batalagoda RRDC.

Cultivar	Total Amino acid content (g/kg), dry basis (n = 3)																	
	Asp	Glu	Ser	His	Gly	Thr	Arg	Ala	Tyr	Val	Met	Trp	Phe	Ise	Leu	Lys	Pro	Total
Dikwee	Mean	6.2 ^e	12.0 ^d	5.3 ^e	1.3 ^e	5.7 ^g	1.8 ^g	3.4 ^e	7.8 ^f	2.0 ^c	1.0 ^b	1.1 ^a	5.6 ^c	3.5 ^e	6.6 ^e	3.4 ^e	3.5 ^c	78.2 ^g
	SD	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.2	0.1	0.2	2.0
Godaheenati	Mean	7.2 ^d	13.8 ^b	6.3 ^c	1.4 ^{de}	7.4 ^d	2.1 ^d	4.3 ^c	9.8 ^d	2.2 ^{bc}	0.6 ^c	1.2 ^a	5.7 ^c	3.6 ^d	7.9 ^c	4.3 ^{bc}	3.9 ^b	90.2 ^d
	SD	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.1	0.4	0.1	0.1	0.05	0.1	0.1	1.5
Masuran	Mean	7.5 ^c	14.2 ^a	6.5 ^{bc}	1.6 ^{bc}	7.8 ^c	2.3 ^c	4.1 ^c	10.2 ^c	2.2 ^{bc}	1.3 ^b	0.7 ^b	5.9 ^b	3.8 ^b	8.2 ^b	4.5 ^b	2.9 ^d	92.6 ^c
	SD	0.1	0.1	0.1	0.04	0.1	0.1	0.1	0.1	0.1	0.02	0.1	0.2	0.1	0.1	0.1	0.2	1.5
Pachchaperumal	Mean	6.9 ^d	13.0 ^c	6.0 ^d	1.5 ^{bcd}	7.1 ^e	1.9 ^e	3.8 ^d	8.7 ^e	2.3 ^b	1.1 ^b	0.8 ^b	5.8 ^{bc}	3.7 ^c	6.7 ^e	3.7 ^d	4.3 ^a	85.1 ^d
	SD	0.2	0.2	0.1	0.1	0.2	0.03	0.1	0.1	0.2	0.1	0.04	0.1	0.1	0.04	0.1	0.1	2.1
Suduru Samba	Mean	7.9 ^b	14.2 ^a	6.7 ^b	1.6 ^b	8.7 ^b	2.3 ^b	4.5 ^b	10.5 ^b	2.1 ^{bc}	1.2 ^b	1.0 ^a	5.7 ^{bc}	3.6 ^d	8.1 ^{bc}	4.5 ^b	4.5 ^a	96.0 ^b
	SD	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.04	0.1	0.1	0.1	0.1	0.3	0.1	2.0
Suduheenati	Mean	6.3 ^e	11.7 ^d	5.4 ^e	1.4 ^{cde}	6.5 ^f	1.9 ^f	3.6 ^{de}	8.7 ^e	2.0 ^c	1.3 ^b	1.1 ^a	5.4 ^d	3.2 ^f	7.0 ^d	4.2 ^c	3.6 ^c	80.4 ^f
	SD	0.2	0.1	0.2	0.1	0.05	0.1	0.1	0.1	0.1	0.2	0.2	0.2	0.1	0.1	0.1	0.1	2.2
Suwanda Samba	Mean	10.7 ^a	14.2 ^a	9.0 ^a	2.3 ^a	11.4 ^a	2.9 ^a	6.3 ^a	10.6 ^a	2.9 ^a	1.7 ^a	0.7 ^b	8.5 ^a	5.4 ^a	10.7 ^a	5.7 ^a	4.3 ^b	117.2 ^a
	SD	0.2	0.1	0.2	0.1	0.1	0.02	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.2	0.1	0.2	1.5

Values within a column followed by different letters are significantly different at $p < 0.05$ according to Duncan Multiple Range test; SD: Standard Deviation; RRDC: Rice Research Development; n = Number of replicates; Asp: Aspartic acid; Glu: Glutamic acid; Ser: Serine; His: Histidine; Gly: Glycine; Lys: Lysine; Thr: Threonine; Arg: Arginine; Ala: Alanine; Tyr: Tyrosine; Val: Valine; Pro: Proline; Met: Methionine; Trp: Tryptophan; Phe: Phenylalanine; Ile: Isoleucine; Leu: Leucine

Table 6: Total amino acid contents of traditional rice cultivars grown in Bombuwala RRDC.

Cultivar	Total Amino acid content (g/kg), dry basis (n = 3)																		
	Asp	Glu	Ser	His	Gly	Thr	Arg	Ala	Tyr	Val	Met	Trp	Phe	Ise	Leu	Lys	Pro	Total	
Dikwee	Mean	8.4 ^b	17.5 ^b	7.3 ^b	2.0 ^a	8.6 ^c	2.6 ^a	4.6 ^b	10.3 ^b	2.3 ^a	8.7 ^{ab}	1.3 ^b	0.7 ^c	6.5 ^a	4.6 ^a	7.9 ^a	4.3 ^a	4.6 ^b	102.1 ^b
	SD	0.05	0.2	0.01	0.1	0.1	0.1	0.02	0.03	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.03	0.04	0.3
Godaheenati	Mean	7.2 ^e	13.6 ^f	5.9 ^f	1.9 ^b	7.2 ^e	2.1 ^e	3.8 ^e	8.5 ^e	2.0 ^b	6.0 ^e	1.4 ^b	0.6 ^c	5.9 ^d	3.8 ^c	6.8 ^f	3.3 ^c	4.1 ^c	84.1 ^f
	SD	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.03	0.1	0.04	0.1	0.02	0.1	0.1	0.8
Masuran	Mean	6.4 ^f	12.7 ^g	5.5 ^g	1.7 ^b	6.6 ^f	2.0 ^e	3.7 ^f	8.1 ^f	2.0 ^b	5.6 ^f	1.3 ^b	0.9 ^a	5.8 ^e	3.4 ^d	6.5 ^g	3.4 ^{bc}	3.9 ^d	79.5 ^g
	SD	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.7
Pachchaperumal	Mean	7.5 ^d	15.2 ^e	6.5 ^e	1.9 ^b	7.6 ^d	2.2 ^d	4.1 ^d	9.1 ^d	2.0 ^b	7.7 ^c	1.8 ^a	0.7 ^c	6.1 ^c	3.9 ^c	7.0 ^e	3.5 ^b	4.6 ^b	91.4 ^e
	SD	0.04	0.03	0.1	0.1	0.1	0.02	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.03	0.04	0.1	0.1	0.5
Suduru Samba	Mean	8.8 ^a	17.6 ^a	7.5	2.1 ^a	9.3 ^a	2.5 ^{ab}	4.9 ^a	10.5 ^a	2.2 ^a	8.8 ^a	1.9 ^a	0.8 ^b	6.4 ^b	4.2 ^b	7.7 ^b	3.4 ^{bc}	5.1 ^a	103.7 ^a
	SD	0.2	0.1	0.1	0.2	0.01	0.04	0.02	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.5
Suduheeanti	Mean	8.7 ^a	16.8 ^c	7.1 ^c	2.1 ^a	8.9 ^b	2.4 ^{bc}	4.5 ^b	10.3 ^b	2.1 ^{ab}	8.7 ^b	1.4 ^b	0.9 ^a	6.5 ^{ab}	4.2 ^b	7.6 ^c	3.5 ^b	3.8 ^d	99.6 ^c
	SD	0.03	0.1	0.1	0.2	0.1	0.1	0.1	0.01	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.01	0.1	0.5
Suwanda Samba	Mean	7.8 ^c	16.2 ^d	6.8 ^d	1.9 ^b	8.6 ^c	2.3 ^c	4.4 ^c	10.1 ^c	2.3 ^a	7.2 ^d	1.4 ^b	0.7 ^c	6.4 ^b	4.2 ^b	7.4 ^d	2.2 ^d	4.7 ^b	94.5 ^d
	SD	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.1	0.1	0.1	0.7

Values within a column followed by different letters are significantly different at $p < 0.05$ according to Duncan Multiple Range test; SD: Standard Deviation; RRDC: Rice Research Development; n = Number of replicates; Asp: Aspartic acid; Glu: Glutamic acid; Ser: Serine; His: Histidine; Gly: Glycine; Lys: Lysine; Thr: Threonine; Arg: Arginine; Ala: Alanine; Tyr: Tyrosine; Val: Valine; Pro: Proline; Met: Methionine; Trp: Tryptophan; Phe: Phenylalanine; Ile: Isoleucine; Leu: Leucine

the amino acid composition of rice. Studies have revealed that, with increasing temperatures of the environment, notable decrease in protein yield and amino acid content in rice [38] and protein content in wheat [39] have been reported. In addition, similarly as observed with this study, Oh, et al. (2019) [40] has pointed out that the amino acid composition is significantly affected by the rice variety and the location of cultivation. Therefore, these findings emphasize the significance of further investigations in this direction involving harmonization of genetic facts and agro climatic practices in breeding techniques for development of rice crops with optimum nutritional quality.

Conclusion

The study presents the validation data of a quantitative method for analysis of amino acids in rice using reversed phase HPLC with diode array detection. The validated method is accurate, precise and complies with the acceptance criteria required in the method validation guidelines. Hence, the method will serve as a reliable tool to evaluate the amino acid composition in rice and other cereal matrices to evaluate its nutritional value in terms of protein quality and for future investigation on breeding related to improvement of protein quality of cereal crops.

Conflict of Interest Statement

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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