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Analytical Methods

# Validation of a reversed-phase high-performance liquid chromatographic method for the determination of free amino acids in rice using L-theanine as the internal standard

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ABSTRACT

The study presents the validation results of the method carried out for analysis of free amino acids (FAAs) in rice using L-theanine as the internal standard (IS) with *o*-phthalaldehyde (OPA) reagent using high-performance liquid chromatography-fluorescence detection. The detection and quantification limits of the method were in the range 2–16 μmol/kg and 3–19 μmol/kg respectively. The method had a wide working range from 25 to 600 μmol/kg for each individual amino acid, and good linearity with regression coefficients greater than 0.999. Precision measured in terms of repeatability and reproducibility, expressed as percentage relative standard deviation (% RSD) was below 9% for all the amino acids analyzed. The recoveries obtained after fortification at three concentration levels were in the range 75–105%. In comparison to L-norvaline, findings revealed that L-theanine is suitable as an IS and the validated method can be used for FAA determination in rice.



## 1. Introduction

Rice, the seed of the grass species *Oryza sativa* (Asian rice) or *Oryza glaberrima* (African rice) contributes to 27% of the world's dietary energy supply (Kennedy, Burlingame, & Nguyen, 2002). It is the most widely consumed staple food in the world including Sri Lanka. Rice accounts for 38.4% percent of the total dietary energy supply while contributing to 37.0% of the dietary protein and 2.7% of the dietary fat intake in the Sri Lankan diet (Kennedy et al., 2002). The nutrient composition of rice differs significantly among varieties (Chandrasekhar & Mulk, 1970). Factors such as agricultural practices, the cultivar, postharvest conditions and handling can have an impact on the nutrient content of rice. Further traditional breeding techniques and genetic engineering of rice cultivars can significantly alter the nutrient composition of rice (Kamara, Konishi, Sasanuma, & Abe, 2010). It has shown that, Asian rice varieties are reported to possess the greatest overall variation in protein content ranging from 4% to 14% (Juliano & Villareal, 1993).

The carbohydrates and proteins being the major constituents, the polished rice grains consist minor amounts of lipids, fiber, sugars and free amino acids (FAAs). It has been reported that the concentration of FAAs together with soluble sugars significantly contribute to the palatability of cooked rice as sensory active flavor agents (Kamara et al., 2010). This direct link of FAAs to the taste of

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rice has created growing interest among scientific community in researching on the levels of FAAs in rice. Consequently, there is a demand for accurate and sensitive analytical methods for the determination of FAAs, which should be suitable for analysis across the micromolar range. However even though there are several studies done on FAAs in other grains (Kovacs et al., 2011; Mustafa, Aman, Andersson, & Eldin, 2007; Nimbalkar, Pai, Pawar, Oulkar, & Dixit, 2012), very limited studies are reported on the FAAs in Asian *Indica* rice varieties (Kamara et al., 2010) and specifically investigations on method performance characteristics related to method validation carried out on FAA determination in rice.

There are several methods described in literature for analysis of free amino acids. In general, the FAAs are extracted after deproteinization with solvents acidified with HCl (IS/1S0, 2005), formic acid (Nimbalkar et al., 2012) or trichloroacetic acid (Kovacs et al., 2011) followed by centrifugation or filtration to remove the extracted particles before injection into the chromatographic systems. The most frequently used solvents are either hot or cold water and ethanol or methanol water mixtures (Mustafa et al., 2007).

Several techniques have been discussed in literature for the detection of amino acids including high performance liquid chromatography (HPLC) that couples diode array, mass spectrometry and fluorescence detection (Henderson, Ricker, & Cliff, 2000), gas chromatography with mass spectrometry (Kaspar, Dettmer, Gronwald, & Oefner, 2008), ion exchange chromatography (Houpert, Tarallo, & Siest, 1975) and capillary electrophoresis with ultra violet-visible or fluorescence detection. With the modern advances in technology, the greater sensitivity that can be achieved with detection techniques such as fluorescence and mass spectrometry, rapid analysis time and the minor sample quantities required, liquid chromatography has become the most popular choice of quantification for amino acid analysis. Due to the sensitivity, high throughput and the simplicity, HPLC analysis which couples automated pre-column derivatization has gained wide acceptance and several derivatizing agents including OPA, (Schwarz, Roberts, & Pasquali, 2005), 9-fluorenyl-methyl chloroformate (FMOC-Cl) (Igor, Krstovic, Glamocic, Jaksic, & Abramovic, 2013), 5-dimethylaminonaphthalene-1-sulfonyl (dansyl) chloride (Vans & Zaerr, 1990), 4-dimethylaminoazobenzene-4-sulfonyl (dabsyl) chloride (Mey et al., 2012) and phenylisothiocyanate (PITC) (Kamara et al., 2010) are used for amino acid analysis. The relatively easier derivatization and the faster reaction at room temperature have made OPA the popular choice for derivatization. In this study, the FAAs in rice after extraction were derivatized with OPA for fluorescence detection.

In general, amino acid quantification is carried out using the internal standard (IS) method. There is an extensive variability in chemical characteristics seen among the amino acids. Basically amino acids can be categorized as polar or as non-polar. The alkyl group attached to the amide group varies from neutral, acidic to basic as well as aliphatic to aromatic. The amino acids can also be categorized based on their hydrophobicity or their hydrophilic nature. In the quantification involved with IS calibration technique, the correct choice of the IS which has the closest resemblance to the chemical characteristics such as the molecular structure, functional groups, polarity *etc.* of the analytes of interest is paramount to the accurate and selective determination. Therefore, use of a single IS to mimic the entire chemistry represented by the range of whole amino acids is not a rational analytical approach as it can actually skew the recoveries for the overall amino acid profile. Generally, best ISs are the isotopic analogs of target analytes. However when detectors other than MS are used, poor separation of isotopic isomers generally encountered in chromatography and the high cost often impede isotopic standards from being considered for everyday use as IS's. Apart from the isotopic analogs, there are very few IS's available which find application in the analysis of amino acids such as L-norleucine (Bedner, Sander, & Sharpless, 2010), L-norvaline (Henderson et al., 2000), 3-nitrotyrosine (Bartolomeo & Maisano, 2006), L-homo-arginine (Bruckner & Westhauser, 2003) *etc.* According to literature, L-norleucine and L-norvaline are the most frequently used, popular choices as IS's in amino acid. L-norleucine is found in the castor seed (*Ricinus communis*) and in certain bacterial strains in nature (Kisumi, Sugiura, Kato, & Chibata, 1976) whereas L-norvaline is reported in the black current buds (*Ribes nigrum*), casein, globin and steerhorn (Limaye et al., 1995), and also found in the antifungal peptide of *Bacillus subtilis* (Nandi & Sen, 1953) and some recombinant proteins found in *E. coli* (Soini et al., 2008). In addition L-norvaline is incorporated in nutritional supplements as an arginase enzyme inhibitor. L-Homo-arginine is observed in grass pea (*Lathyrus sativus L.*) as reported by Piergiovanni and Damascelli (2011) and found in maternal plasma during pregnancy (Tsikas & Wu, 2015), in tissue of biological samples (May et al., 2015) and also recognized as an indicator of cardiovascular risk factor (Kayacelebi, Beckmann, & Gutzki, 2014). In addition, 3-nitrotyrosine is an amino acid recognized as an indicator of cell damage and inflammation (Ashan, 2013). The occurrence of these particular amino acids in biological samples and nutritional supplements as in the case of L-norvaline impedes their use as IS's in quantification.

In this scenario, there is an urge for analytical research focusing on alternative IS's which provide better representation of the chemistry inherent by the amino acids as well as IS's which claim uniqueness/distinctiveness in nature.

L-Theanine (*N*-ethyl-L-glutamine) is a unique non-protein amino acid found in plants of *theaceae* family, first discovered in tea leaves (Sakato, 1949). L-theanine accounts for the unique taste characteristic of tea known as "umami" (Yamaguchi & Ninomiya, 2000) and has evidenced in acting as an antagonist against paralysis induced by caffeine, as a neurotransmitter in the brain as well as causing relaxation-inducing effect in humans (Chu et al., 1997) and hence has been comprehensively studied in the areas related to food science and human nutrition. Apart from the plants of *theaceae*, theanine has been reported from a *basidiomycetes* fungus *Xerocomus badius* (Casimir, Jadot, & Renard, 1960). This uniqueness, warrants its use as an IS in the analysis of amino acids in other food matrices except which originates from *theaceae* family, *Xerocomus badius* and theanine incorporated nutritional supplements. Further, the chemical stability and the better chromatographic resolution stand out as few of the major advantages which deems L-theanine to be considered as an IS.

The present work reports the procedures implemented in the validation process for quantitation of FAAs in rice with the use of L-theanine as the IS in comparison to L-norvaline and the first extensive study ever reported on analysis of FAAs in Sri Lankan traditional rice varieties and the findings of the study will provide a valuable contribution to the very limiting data available on a comprehensive analytical method validation work carried out on FAA analysis in grains and specifically in rice. Therefore this study will provide an insight as to use of L-theanine as an IS in amino acid analysis and the analysis of FAAs using this validated method will provide important reference in finding the relationship among free amino acid profile, taste and the biological activity reported in rice varieties.

## 2. Materials and methods

### 2.1. Materials

Amino acid reference standards; L-aspartic acid (Asp), L-serine (Ser), L-glutamic acid (Glu), L-glutamine (Gln), glycine (Gly),

L-histidine (His), L-asparagine (Asn), L-arginine (Arg), L-threonine (Thr), L-alanine (Ala), L-tyrosine (Tyr), L-valine (Val), L-methionine (Met), L-isoleucine (Ile), L-tryptophan (Trp), L-leucine (Leu), L-phenylalanine (Phe) and L-norvaline (Nva), each of purity >98% were obtained from Sigma Aldrich Chemicals, St. Louis, MO. The internal standard, L-theanine of purity >98% was purchased from Baxter Smith Labs, USA. L-Asn, L-Gln, L-Trp, L-theanine and L-Nva were prepared in ultra pure water while the rest of the amino acids were prepared in 0.1 M HCl solution.

The derivatizing agent, *o*-phthalaldehyde 3-mercaptopropionic acid (OPA-3MPA) and borate buffer (pH-10.2) were from Agilent. All the other chemicals used were of analytical reagent grade and the solvents were of HPLC grade purchased from Sigma Aldrich. The mobile phases, A (40 mmol/L $Na_2HPO_4$ at pH 7.8), B (45% acetonitrile, 45% methanol, 10% water) were prepared freshly on each day prior to analysis.

The traditional rice samples were obtained from the rice research development centers (RRDC) at Bathalegoda and Bombuwala in Sri Lanka.

### 2.2. Sample preparation

The finely ground rice samples were sieved through 0.5 mm sieve. The free amino acids in the samples were extracted by adding, to 2.0 g of the sieved rice sample, 10.0 mL of thiodiglycol acidified with HCl such that its concentration of HCl is 0.1 mol/L in the final extraction solution. To each extraction tube, 100 μL of the internal standards, L-theanine and L-norvaline each of 10 μmol/mL concentration were added. Then the free amino acids in the samples were extracted by shaking the mixture in a mechanical shaker at 125 rpm for 20 min. Then to 5.0 mL of the extracted mixture, 2.5 mL of 5-sulfosalicylic acid was added to precipitate the co-extracted macromolecules like proteins by shaking in an ultrasonic mixer for 5 min. Then to 5.0 mL of the supernatant solution, 1 M NaOH solution was added to adjust the pH of the mixture to pH – 2.2, finally being made up to a total volume of 10 mL in a volumetric flask by adding citrate buffer having pH of 2.2. The solution was injected into a HPLC system after filtering through 0.22 μm syringe filter.

### 2.3. Instrumentation and analytical HPLC method

The analysis was performed using an Agilent 1100 HPLC systems (Agilent Technologies, Palo Alto, CA). The system consisted of a quaternary pump (G1311A), a fluorescence detector (G1321A) and an auto sampler (G1313A). An Agilent Zorbax Eclipse AAA column (4.6 mm × 150 mm, 5 μm) was used for the chromatographic separation. The gradient elution started with 100% A for 0 min; ramped to 88% A within next 8 min; ramped to 70% A till 35 min; ramped to 55% A till 37 min; ramped to 100% A till 43 min and kept 100% A till 45 min. The column was operated at 40 °C and the flow rate of the method was set at 2 mL/min throughout the runtime.

The standards and samples were also run on a Phenomenex Gemini column (4.6 mm × 150 mm, 5 μm) using the same mobile phases for assessing the robustness and ruggedness of the method. The gradient elution started with 100% A at 0 min; ramped to 59.5% A within 40.5 min; ramped to 39% A till 41 min; kept at 39% A till 44 min; ramped to 18% A, till 44.5 min; ramped to 100% A till 46.5 min and kept 100% till 49 min. The column compartment was operated at 40 °C and the flow rate of the method was set at 1 mL/min. Zorbax AAA guard columns (4.6 mm × 12.5 mm) from Agilent USA were used to protect the columns.

Automated pre-column derivatization with OPA-3MPA was performed according to the injector program as listed in Table 1 (Henderson et al., 2000). The derivatized FAAs were detected by fluorescence detector with excitation and emission at 340 nm, 450 nm respectively with a photo multiplier tube (PMT) gain of 10. Agilent Chemstation software was used for data acquisition and analysis.

### 2.4. Validation of the method

The method was validated as per the guidelines described in FDA, AOAC International and Eurachem Method validation guidelines (AOAC, 2002; EURACHEM Guide, 2014; FDA, 2012). In order to evaluate the method performance characteristics, the samples were fortified with the analytes at three concentration levels (125, 225 and 450 μmol/kg) covering the working range. Each concentration was analyzed separately in six replicates. Similarly, limit of detection (LOD), limit of quantitation (LOQ) values were evaluated by analyzing the blanks fortified at the lowest detection levels.

## 3. Results and discussion

The challenge faced in any quantitative analysis is to accurately determine the concentration of target analytes in the original sample. Often there is an involvement for preparation of the samples prior to injection into the instrument of interest introducing additional errors from many variables involved with sample preparation. With the use of an IS, these variations introduced to volume can be accounted. Further when an IS is used, since a signal-ratio of analyte to IS is used for quantification, a moderate proportional matrix effect can be corrected. In addition, in chromatographic analysis, through the use of an IS, variations arising from fluctuations in flow rates, temperatures, detector response occurring between runs which affect the response of the analyte of interest are compensated. Therefore IS calibration provides better accuracy over the external calibration method. However the correct choice of the IS which has the closest resemblance to the chemical characteristics such as the molecular structure, functional groups, polarity *etc.* of the analytes of interest is critical in the quantification involved with IS calibration technique.

The amino acids represent a myriad of chemical characteristics based on the alkyl side chain attached. Lys, Arg, Asp and Glu belong to the category of amino acids with a charged alkyl side chain

**Table 1**
Injector program.

| Line | Function | Amount | Reagent |
|---|---|---|---|
| 1 | Draw | 2.5 μL | Borate buffer |
| 2 | Draw | 0.5 μL | Sample |
| 3 | Mix | 3.0 in air max speed 2 times | |
| 4 | Wait | 0.5 min | |
| 5 | Draw | 0 μL | Water – (needle wash using uncapped vial) |
| 6 | Draw | 0.5 μL | OPA |
| 7 | Mix | 3.5 μL in air Max speed 6 times | |
| 8 | Draw | 0 μL | Water (needle wash using water in uncapped vial |
| 9 | Draw | 32 μL | Water (capped vial) |
| 10 | Mix | 18 μL in air max speed 2 times | |
| 11 | Inject | | |

while Ser, Thr, Asn, Gln, His and Tyr possess hydrophilic alkyl side chains. The hydrophobic amino acids include Ala, Val, Leu, Ile, Pro, Phe, Trp, and Met.

In amino acid analysis, L-norvaline and L-norleucine are the overwhelming choice of IS's found in literature and are hydrophobic amino acids. Therefore, the use of these two as IS's would not serve the purpose intended by an IS for hydrophilic amino acids as the behavior of hydrophilic compounds are not represented by the two IS's. Further L-norvaline and L-norleucine elute near the end of many typical chromatographic runs. Therefore, by the use of the two IS's, the correction for signal response due to fluctuations experienced by the early eluting amino acids in a chromatographic run are not achieved. However, L-theanine is a hydrophilic amino acid and elutes in the middle of typical chromatographic runs with better resolution from the rest of the amino acids. Therefore, for early eluting and hydrophilic amino acids, the role intended from an IS is better achieved with L-theanine compared to L-norvaline or L-norleucine.

The validation data obtained for method performed using L-theanine and L-norvaline as IS's are listed in Table 2 and Table 3.

### 3.1. Selectivity

The resolution factors (Rs) calculated for all the amino acids were greater than 1 with the lowest Rs values obtained for Gly/Thr and Ile/Phe demonstrating excellent resolution, indicated better selectivity for all the amino acids analyzed as given in Fig. 1. The relative retention times calculated with respect to IS demonstrated the slightest variations with less than 0.1% relative standard deviation (RSD) among batches of analysis providing better selectivity for the analyzed amino acids.

### 3.2. Precision

The precision of the method was measured under repeatable conditions on samples that contain targeted analytes spiked at three concentration levels (125, 225 and 450 μmol/kg) covering the working range based on six replicate analysis on the same day. Similarly the intermediate precision was calculated based on six replicate analysis carried out at each concentration level separately on different time periods by different analysts in a random order.

The precision of the method expressed as percentage of the relative standard deviation (% RSD) of concentrations, measured using repeatability and intermediate precision conditions mentioned above were below 9% for all the amino acids analyzed as given in Table 2.

### 3.3. Accuracy and recovery

The accuracy was evaluated based on recovery due to the unavailability of a certified reference material on FAAs. Recovery was evaluated after spiking at three concentration levels (125, 225 and 450 μmol/kg). The recoveries obtained were in the range 75–105%. Trp reported the overall lowest recovery values and this was in agreement with the method validation studies carried out on free amino acids in cereals by Mustafa et al. (2007). However the overall recoveries obtained in the present study for Glu are well within the acceptable recovery range compared to the recovery values reported by Mustafa et al., for Glu in all the grain samples studied. Except for Trp, Val, Gly and Thr, recoveries were above 80% for all the amino acids tested in the entire working range, (Table 2). Therefore, for the majority of the amino acids studied, the recovery values were within 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 (FDA, 2012).

### 3.4. Limit of detection and Limit of quantitation

To the mean value obtained for the blank response fortified at lowest detectable concentrations, the LODs and the LOQs were

**Table 2**
Precision and recovery.

| Amino Acid | RSD (%) n = 6 Internal standard | | | | | | Recovery (%) ± SD n = 6 Internal standard | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Theanine | | | Norvaline | | | Theanine | | | Norvaline | | |
| | μmol/kg | | | | | | μmol/kg | | | | | |
| | 125 | 225 | 450 | 125 | 225 | 450 | 125 | 225 | 450 | 125 | 225 | 450 |
| Asp | 5.2 | 4.7 | 3.0 | 6.0 | 6.0 | 7.0 | 88 ± 5 | 96 ± 5 | 105 ± 3 | 91 ± 7 | 91 ± 6 | 104 ± 7 |
| Glu | 5.1 | 5.1 | 4.0 | 5.0 | 6.0 | 2.0 | 96 ± 5 | 90 ± 5 | 102 ± 4 | 99 ± 5 | 87 ± 5 | 98 ± 2 |
| Asn | 6.0 | 3.0 | 1.4 | 4.0 | 7.0 | 4.0 | 103 ± 6 | 86 ± 5 | 87 ± 1 | 108 ± 4 | 84 ± 4 | 90 ± 4 |
| Ser | 4.6 | 1.3 | 3.3 | 3.0 | 7.0 | 6.0 | 88 ± 5 | 98 ± 1 | 98 ± 3 | 85 ± 3 | 97 ± 4 | 101 ± 6 |
| Gln | 2.8 | 3.1 | 3.0 | 3.0 | 6.0 | 3.0 | 87 ± 3 | 79 ± 3 | 93 ± 3 | 91 ± 3 | 76 ± 3 | 95 ± 3 |
| His | 4.3 | 8.0 | 4.0 | 6.0 | 8.0 | 3.0 | 100 ± 4 | 93 ± 8 | 84 ± 4 | 101 ± 6 | 99 ± 8 | 88 ± 3 |
| Gly | 4.2 | 1.7 | 3.0 | 6.0 | 4.0 | 2.0 | 89 ± 4 | 95 ± 2 | 95 ± 3 | 83 ± 6 | 95 ± 5 | 92 ± 2 |
| Thr | 4.0 | 3.0 | 2.0 | 3.0 | 3.0 | 2.0 | 93 ± 4 | 77 ± 3 | 100 ± 2 | 97 ± 2 | 77 ± 3 | 100 ± 2 |
| Arg | 6.0 | 2.1 | 3.9 | 7.0 | 2.0 | 1.0 | 85 ± 6 | 86 ± 2 | 95 ± 4 | 85 ± 7 | 85 ± 2 | 98 ± 1 |
| Ala | 4.3 | 3.0 | 4.1 | 7.0 | 3.0 | 4.0 | 87 ± 4 | 91 ± 3 | 95 ± 4 | 86 ± 7 | 88 ± 3 | 92 ± 4 |
| Tyr | 1.8 | 1.6 | 2.9 | 2.0 | 1.0 | 3.0 | 83 ± 2 | 92 ± 2 | 95 ± 3 | 86 ± 2 | 92 ± 1 | 97 ± 3 |
| Val | 2.4 | 0.5 | 3.6 | 3.0 | 0.6 | 5.0 | 78 ± 2 | 89 ± 1 | 93 ± 4 | 79 ± 3 | 88 ± 1 | 95 ± 5 |
| Met | 2.9 | 1.4 | 2.0 | 2.0 | 1.0 | 6.0 | 98 ± 3 | 98 ± 1 | 99 ± 1 | 98 ± 2 | 96 ± 1 | 100 ± 3 |
| Trp | 4.0 | 3.4 | 1.9 | 3.0 | 2.0 | 1.0 | 76 ± 4 | 75 ± 3 | 78 ± 2 | 72 ± 3 | 77 ± 2 | 77 ± 1 |
| Phe | 2.0 | 3.0 | 4.0 | 2.0 | 2.0 | 7.0 | 96 ± 2 | 94 ± 3 | 104 ± 5 | 96 ± 2 | 94 ± 2 | 107 ± 7 |
| Ile | 4.0 | 2.0 | 4.0 | 3.0 | 1.0 | 7.0 | 89 ± 4 | 99 ± 2 | 104 ± 4 | 91 ± 3 | 98 ± 1 | 105 ± 7 |
| Leu | 1.0 | 1.8 | 0.9 | 6.0 | 1.0 | 4.0 | 99 ± 1 | 102 ± 2 | 99 ± 1 | 105 ± 6 | 100 ± 1 | 99 ± 4 |

RSD: Relative Standard Deviation.
SD: Standard Deviation.
n = Number of replicates.
Asp: Aspartic acid, Glu: Glutamic acid, Asn: Asparagine, Ser: Serine, Gln: Glutamine, His: Histidine, Gly: Glycine, Thr: Threonine, Arg: Arginine, Ala: Alanine, Tyr: Tyrosine, Val: Valine, Met: Methionine, Trp: Tryptophan, Phe: Phenylalanine, Ile: Isoleucine, Leu: Leucine.

**Table 3**
Linearity, LOD and LOQ.

| Amino Acid | Internal Standard | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Theanine | | | | Norvaline | | | |
| | Calibration equation | $R^2$ | LOD µmol/kg | LOQ µmol/kg | Calibration equation | $R^2$ | LOD µmol/kg | LOQ µmol/kg |
| Asp | y = 7.98E−04x + 8.51E−03 | 0.999 | 9 | 9 | y = 8.18E−04x + 2.37E−03 | 0.999 | 12 | 13 |
| Glu | y = 7.22E−04x + 7.26E−03 | 0.999 | 10 | 12 | y = 7.41E−04x + 1.70E−03 | 0.999 | 10 | 12 |
| Asn | y = 9.20E−04x + 5.23E−03 | 0.999 | 7 | 8 | y = 9.44E−04x − 1.81E−03 | 0.999 | 7 | 8 |
| Ser | y = 8.74E−04x + 3.22E−02 | 0.999 | 15 | 17 | y = 8.97E−04x + 2.51E−02 | 0.999 | 14 | 16 |
| Gln | y = 8.21E−04x + 9.30E−03 | 0.999 | 4 | 5 | y = 8.42E−04x + 3.00E−03 | 0.999 | 4 | 5 |
| His | y = 5.24E−04x − 1.47E−03 | 0.999 | 12 | 13 | y = 5.37E−04x − 5.41E−03 | 0.999 | 12 | 13 |
| Gly | y = 8.37E−04x + 2.47E−02 | 0.999 | 16 | 19 | y = 8.59E−04x + 1.80E−02 | 0.999 | 19 | 20 |
| Thr | y = 8.53E−04x + 7.78E−03 | 0.999 | 9 | 10 | y = 8.75E−04x + 1.23E−03 | 0.999 | 8 | 9 |
| Arg | y = 9.94E−04x + 1.43E−02 | 0.999 | 11 | 13 | y = 1.02E−03x + 6.62E−03 | 0.999 | 11 | 12 |
| Ala | y = 8.62E−04x + 1.45E−02 | 0.999 | 11 | 12 | y = 8.84E−04x + 7.79E−03 | 0.999 | 10 | 11 |
| Tyr | y = 9.06E−04x + 2.71E−03 | 0.999 | 12 | 14 | y = 9.29E−04x − 4.16E−03 | 0.999 | 12 | 13 |
| Val | y = 1.00E−03x + 2.49E−02 | 0.999 | 2 | 3 | y = 1.03E−03x + 1.70E−02 | 0.999 | 2 | 3 |
| Met | y = 9.94E−04x + 6.45E−03 | 0.999 | 7 | 7 | y = 1.02E−03x − 1.16E−03 | 0.999 | 6 | 7 |
| Trp | y = 8.36E−04x + 1.17E−02 | 0.999 | 5 | 6 | y = 8.58E−04x + 5.16E−03 | 0.999 | 5 | 5 |
| Phe | y = 9.87E−04x − 3.75E−03 | 0.999 | 9 | 10 | y = 1.01E−03x − 1.11E−02 | 0.999 | 12 | 13 |
| Ile | y = 1.01E−03x + 3.20E−04 | 0.999 | 16 | 18 | y = 1.04E−03x − 7.29E−03 | 0.999 | 16 | 17 |
| Leu | y = 1.02E−03x + 3.46E−03 | 0.999 | 14 | 15 | y = 1.04E−03x − 4.25E−03 | 0.999 | 13 | 15 |

LOD: Limit of Detection.
LOQ: Limit of Quantification.
$R^2$: Regression Coefficient.
Asp: Aspartic acid, Glu: Glutamic acid, Asn: Asparagine, Ser: Serine, Gln: Glutamine, His: Histidine, Gly: Glycine, Thr: Threonine, Arg: Arginine, Ala: Alanine, Tyr: Tyrosine, Val: Valine, Met: Methionine, Trp: Tryptophan, Phe: Phenylalanine, Ile: Isoleucine, Leu: Leucine.

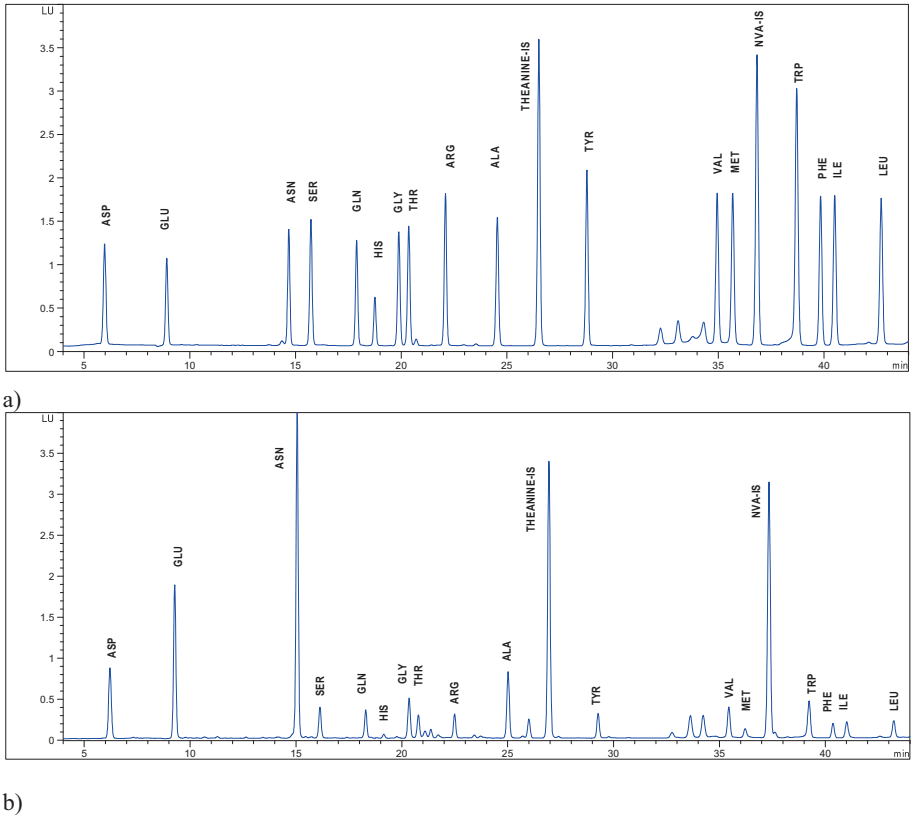


**Fig. 1.** HPLC Chromatograms. a) Amino acids standard mixture. b) Free amino acids in a rice sample.

evaluated by adding approximately 3 times and 5 times of the standard deviation of the response detected for the fortified blank at the lowest detectable concentrations respectively. The LODs and the LOQs for the method were in the range 2.0–16.0 µmol/kg and 3–19 µmol/kg respectively allowing high sensitivity in detection of FAAs in the micromolar levels. The lowest LOD and LOQ values observed were for Val whereas the highest LOQ observed was for Gly, the amino acid with the smallest molecular mass as given in Table 3. Achievement of low LOD and LOQ values are particularly important in analysis of free amino acids in rice where amino acids which account significantly for taste are present in minute amounts, usually in micromolar range.

**Table 4**
Working range and the expanded uncertainty of the method.

| Amino acid | Working range µmol/kg | % Expanded Uncertainty (k = 2) |
|---|---|---|
| Asp | 25–600 | 15 |
| Glu | 25–600 | 15 |
| Asn | 25–600 | 16 |
| Ser | 25–600 | 15 |
| Gln | 25–600 | 17 |
| His | 25–600 | 11 |
| Gly | 25–600 | 12 |
| Thr | 25–600 | 14 |
| Arg | 25–600 | 15 |
| Ala | 25–600 | 14 |
| Tyr | 25–600 | 11 |
| Val | 25–600 | 12 |
| Met | 25–600 | 13 |
| Trp | 25–600 | 15 |
| Phe | 25–600 | 10 |
| Ile | 25–600 | 11 |
| Leu | 25–600 | 13 |

Coverage factor (k) = 2.
Asp: Aspartic acid, Glu: Glutamic acid, Asn: Asparagine, Ser: Serine, Gln: Glutamine, His: Histidine, Gly: Glycine, Thr: Threonine, Arg: Arginine, Ala: Alanine, Tyr: Tyrosine, Val: Valine, Met: Methionine, Trp: Tryptophan, Phe: Phenylalanine, Ile: Isoleucine, Leu: Leucine.

### 3.5. Linearity

The calibration range consisted of six calibration levels at 25, 50, 125, 250, 450 and 600 µmol/kg prepared by fortifying blank solutions and analyzed identically subjecting to the sample preparation procedure. The peak area ratio of standard to IS at each concentration is used to construct a linear regression model. The regression coefficient, $R^2$ determined using peak area ratio of standard to IS at calibration levels demonstrated 0.999 for all amino acids analyzed as given in Table 3.

### 3.6. Measurement uncertainty

The Guide to Expression of Uncertainty in Measurement (JCGM 100, 2008) defines uncertainty as the parameter associated with the result of a measurement, which characterizes the dispersion of the values that could reasonably be attributed to the measurand. The percentage expanded uncertainties calculated for each amino acid as per the guide, considering the factors contributing to the final result with a coverage factor of 2 (k = 2) were below 18% for all the amino acids analyzed as given in Table 4. The major contributions to the final percentage uncertainty were from the uncertainties arising from the repeatability of the method and the regression analysis involved in the calibration step.

### 3.7. Robustness of the method

The recoveries of the amino acids were significantly affected by the extraction speed and the duration of the extraction while the pH of the mobile phase, the flow rate and the equilibration time of the column evaluated using the students *t*-test, demonstrated to cause a significant impact on the resolution of the amino acids during a chromatographic run. Therefore these parameters need to be critically controlled during FAA analysis.

In the extraction step, a variety of components such as proteins and peptides that can interfere with the chromatographic analysis gets co-extracted progressively clogging the column thereby increasing the back pressure degrading the chromatographic

**Table 5**
Free amino acid contents of traditional rice cultivars.

| Cultivar Type | | Free Amino acid content (µmol/100 g), dry basis (n = 6) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Asp | Glu | Asn | Ser | Gln | His | Gly | Thr | Arg | Ala | Tyr | Val | Met | Trp | Phe | Ise | Leu |
| Bombuwala RRDC | | | | | | | | | | | | | | | | | | |
| Dikwee | Mean | 97.5 | 132.4 | 78.6 | 22.8 | 5.4 | 9.8 | 28.5 | 9.7 | 20.6 | 62.5 | 9.4 | 11.6 | 2.7 | 19.0 | 7.9 | 5.0 | 8.2 |
| | SD | 1.3 | 1.7 | 1.4 | 1.8 | 0.4 | 0.6 | 0.7 | 0.2 | 0.9 | 0.7 | 1.1 | 0.4 | 0.2 | 2.7 | 2.5 | 1.6 | 1.0 |
| Godaheenati | Mean | 48.7 | 39.7 | 56.4 | 24.1 | 2.7 | 17.7 | 50.6 | 14.6 | 25.2 | 80.4 | 9.0 | 13.1 | ND* | 69.1 | 7.1 | 6.1 | 9.9 |
| | SD | 0.2 | 1.1 | 1.7 | 2.8 | 1.1 | 0.8 | 1.1 | 0.7 | 0.9 | 0.9 | 0.6 | 0.8 | – | 4.0 | 2.1 | 1.7 | 2.9 |
| Masuran | Mean | 36.0 | 62.0 | 62.3 | 21.9 | 6.0 | 12.0 | 39.4 | 8.9 | 12.0 | 83.1 | 6.6 | 9.1 | ND* | 14.0 | 9.0 | 6.1 | 6.3 |
| | SD | 2.8 | 4.7 | 3.4 | 2.4 | 3.8 | 4.4 | 3.2 | 1.8 | 2.3 | 6.6 | 1.5 | 1.1 | – | 6.4 | 6.9 | 2.9 | 2.2 |
| Pachchaperumal | Mean | 48.9 | 66.0 | 69.0 | 15.2 | 2.3 | 4.7 | 28.0 | 4.8 | 8.0 | 33.6 | 4.5 | 6.1 | 1.1 | 15.1 | 6.9 | 1.8 | 4.4 |
| | SD | 0.3 | 0.9 | 1.4 | 0.8 | 0.3 | 0.9 | 1.5 | 0.4 | 1.2 | 2.8 | 0.5 | 0.4 | 1.2 | 0.7 | 3.4 | 0.9 | 0.4 |
| Sooduru Samba | Mean | 27.1 | 43.6 | 73.2 | 26.0 | 1.5 | 9.6 | 38.1 | 10.4 | 24.0 | 78.8 | 5.8 | 11.0 | 2.0 | 34.2 | 3.8 | 3.6 | 6.4 |
| | SD | 0.5 | 1.2 | 1.0 | 0.9 | 0.3 | 0.2 | 0.6 | 0.2 | 0.5 | 0.9 | 0.1 | 0.5 | 0.1 | 1.4 | 0.5 | 0.4 | 0.5 |
| SuduHeenati | Mean | 71.4 | 109.6 | 221.0 | 22.9 | 3.6 | 15.6 | 45.2 | 8.0 | 33.5 | 54.6 | 9.5 | 12.5 | 1.3 | 27.7 | 3.4 | 3.8 | 6.0 |
| | SD | 1.0 | 1.7 | 3.2 | 1.6 | 0.5 | 0.6 | 0.8 | 0.5 | 0.6 | 1.0 | 0.4 | 0.6 | 1.3 | 1.9 | 0.5 | 0.9 | 0.5 |
| Suwanda Samba | Mean | 63.6 | 118.4 | 72.7 | 41.0 | 6.2 | 5.2 | 40.1 | 2.8 | 16.3 | 87.5 | 8.4 | 12.9 | 2.8 | 28.1 | 6.0 | 5.1 | 7.4 |
| | SD | 0.4 | 1.3 | 4.0 | 2.4 | 0.2 | 1.0 | 0.6 | 0.9 | 1.2 | 2.2 | 1.2 | 3.9 | 3.3 | 2.4 | 2.1 | 1.9 | 2.3 |
| Bathalegoda RRDC | | | | | | | | | | | | | | | | | | |
| Dikwee | Mean | 56.2 | 53.7 | 76.7 | 17.3 | 4.1 | 16.7 | 42.4 | 8.3 | 18.9 | 73.8 | 10.3 | 8.0 | ND * | 31.7 | 8.1 | 1.9 | 5.0 |
| | SD | 1.5 | 1.9 | 1.1 | 1.2 | 0.6 | 0.7 | 0.8 | 0.7 | 0.7 | 1.2 | 1.0 | 0.3 | – | 2.2 | 0.8 | 0.3 | 0.8 |
| Godaheenati | Mean | 25.7 | 55.5 | 48.5 | 15.6 | 5.0 | 15.7 | 38.5 | 7.7 | 14.5 | 78.2 | 8.9 | 8.1 | ND* | 19.1 | 4.7 | 2.1 | 3.7 |
| | SD | 0.2 | 1.1 | 1.7 | 2.8 | 1.1 | 0.8 | 1.1 | 0.7 | 0.9 | 0.9 | 0.6 | 0.8 | – | 0.3 | 0.5 | 0.6 | 0.3 |
| Masuran | Mean | 47.9 | 61.1 | 55.7 | 15.4 | 1.7 | 8.5 | 37.8 | 7.1 | 16.1 | 70.8 | 8.2 | 10.1 | ND* | 14.7 | 2.7 | 3.7 | 6.3 |
| | SD | 1.1 | 1.4 | 1.7 | 0.7 | 0.3 | 0.8 | 1.1 | 0.3 | 0.8 | 2.0 | 0.6 | 1.0 | – | 1.6 | 0.6 | 0.4 | 0.7 |
| Pachchaperumal | Mean | 16.8 | 32.0 | 33.9 | 14.1 | 2.8 | 10.5 | 41.9 | 8.6 | 12.2 | 57.9 | 5.0 | 6.3 | 2.2 | 3.5 | 1.7 | 2.7 | 4.1 |
| | SD | 1.0 | 0.7 | 0.7 | 0.6 | 0.5 | 0.6 | 0.8 | 0.5 | 0.5 | 1.4 | 0.5 | 2.2 | 0.9 | 1.1 | 0.5 | 0.3 | 0.7 |
| Sooduru Samba | Mean | 118.5 | 98.2 | 168.7 | 43.3 | 4.4 | 9.7 | 57.3 | 17.8 | 35.1 | 96.2 | 8.8 | 17.6 | 2.3 | 1.6 | 5.5 | 7.0 | 11.3 |
| | SD | 1.0 | 1.6 | 3.0 | 0.6 | 1.7 | 0.4 | 0.4 | 1.5 | 0.9 | 1.0 | 0.5 | 0.4 | 2.1 | 1.3 | 2.6 | 0.3 | 0.3 |
| SuduHeeanti | Mean | 70.0 | 90.4 | 73.0 | 17.9 | 1.8 | 8.7 | 34.0 | 6.6 | 10.3 | 55.8 | 4.0 | 4.1 | ND* | 1.6 | 1.0 | 1.8 | 2.4 |
| | SD | 1.9 | 3.0 | 2.3 | 1.5 | 0.4 | 1.1 | 1.4 | 0.5 | 0.9 | 1.3 | 0.5 | 0.9 | – | 1.3 | 0.7 | 1.3 | 0.6 |
| Suwanda Samba | Mean | 53.6 | 53.4 | 135.4 | 25.6 | 3.6 | 27.9 | 36.8 | 9.9 | 21.5 | 60.7 | 9.4 | 5.9 | 9.9 | 42.7 | 3.5 | 2.8 | 6.5 |
| | SD | 0.4 | 1.3 | 4.0 | 2.4 | 0.2 | 1.0 | 0.6 | 0.9 | 1.2 | 2.2 | 1.2 | 3.9 | 3.3 | 2.4 | 2.1 | 1.9 | 2.3 |

SD: Standard Deviation, RRDC: Rice Research Development Center, ND: Not Detected, *Limit of Quantification: 0.7 µmol/100g, n = Number of replicates.
Asp: Aspartic acid, Glu: Glutamic acid, Asn: Asparagine, Ser: Serine, Gln: Glutamine, His: Histidine, Gly: Glycine, Thr: Threonine, Arg: Arginine, Ala: Alanine, Tyr: Tyrosine, Val: Valine, Met: Methionine, Trp: Tryptophan, Phe: Phenylalanine, Ile: Isoleucine, Leu: Leucine.

performance by loss of resolution (Nollet, 2000). This was overcome by the deprotenization of the extracted samples achieved by precipitating with sulfosalicylic acid.

Further, due to the tendency of precipitation of the phosphate buffer in the column during the gradient elution steps, washing of the column first using sufficient amounts of water followed by the mixtures of organic and aqueous mobile phases that does not carry buffer after every batch of analysis was performed to protect the integrity of the columns used for the analysis.

The method performance characteristics summarized in this study are among the very limiting analytical validation work carried out on analysis of FAAs in grains and specifically in rice varieties. The findings reveal that the method performance characteristics are in agreement with those specified for specific concentration levels as defined by the FDA in Guidelines for the Validation of Chemical Methods for the FDA Foods Program (FDA, 2012). The statistical analysis done using one way ANOVA evaluated from statistical software (SAS version 9.1), proved that there is no significant difference in results obtained for all validation data produced using L-norvaline as the IS over which obtained using L-theanine at 0.05 probability level. Therefore L-theanine is suitable as an IS and the validated method can be used for determination of FAAs in rice.

The summary of FAAs determined in six traditional rice varieties grown at the Bathalegoda and Bombuwala Rice Research Development Centers of Sri Lanka using the validated method are listed in Table 5. According to the FAA profiles, Glu, Asp, Asn and Ala are the predominant FAAs reported in the experimented rice cultivars while Met being the least present. The findings on total FAA levels are comparable with the very limited research done on FAAs in *Indica* rice varieties in Asia (Kamara et al., 2010), however demonstrate an overall variation among individual amino acids. Further, statistical investigations done using ANOVA on the FAA profiles of each Sri Lankan traditional rice cultivar suggest that the free amino acid composition significantly differ with respect to the cultivar and the geographical location therefore, authenticity and geographical origin can be attributed to the FAA profile. However further studies are required to validate these findings.

## 4. Conclusion

The validated method is reproducible and accurate, allowing determination of FAA composition in rice. The findings will provide a reliable tool to evaluate the FAA composition in rice and scientific guidance on the suitability of L-theanine as an IS in amino acid analysis.

Most importantly, since L-theanine elutes in the middle during the chromatographic run and is a polar amino acid, for polar and early eluting amino acids, L-theanine's role as an IS, is more representative and therefore superior over other conventional internal standards.

The analysis of FAAs using this validated method will provide important reference in finding the relationship among FAA profile, taste and the biological activity in rice.

## Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this publication.

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