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Development and validation of a method for direct, underivatized analysis of free amino acids in rice using liquid chromatography–tandem mass spectrometry

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ABSTRACT

Inspired by the apparent relationship of free amino acids (FAAs) which are present in minute quantities with the organoleptic characteristics of food, there is an increased demand for analytical methods sensitive in trace level detection. This study presents the validation results of a simple and rapid method developed for direct, underivatized analysis of FAAs in rice using liquid chromatography–tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI).

The method demonstrated satisfactory selectivity for twenty FAAs with minimum matrix effect. The recoveries obtained for samples fortified at three concentration levels: low mid and high, covering the working range of the method were in the range 80%–110%. The precision measured in terms of repeatability and reproducibility of the method expressed as percentage relative standard deviation (% RSD) were below 10% for the amino acids analyzed. The detection limits (LODs) and quantification limits (LOQs) of the method were in the range 0.4–1.0 mg/kg and 0.6–1.2 mg/kg respectively. Method had a wide linear range between 1.25–100 mg/kg with regression coefficients greater than 0.999 obtained over seven calibration levels. The method was also found robust over other cereals including corn, wheat and finger millet with satisfactory recoveries and precision values. The percentage expanded uncertainties calculated with the coverage factor of 2 (k = 2), were below 14% for the analyzed amino acids.

The developed, simple and rapid LC–MS/MS method is accurate and reproducible, allowing determination of underivatized FAAs in rice and comply with the international method validation guideline requirements.

1. Introduction

Rice which belongs to the genus *Oryza* is the most widely consumed primary food source for more than half of the world's population [1,2]. As the dietary staple, rice significantly contributes to the total dietary energy supply and the dietary protein intake of the Asian diet [2]. Thousands of varieties of rice are grown around the world with a wide genetic diversity. It has been reported that depending on the cultivar, breeding techniques, agricultural practices and postharvest conditions, the nutrient composition of rice can significantly vary [3,4]. Rice mainly contains carbohydrates and proteins as the major constituents while consisting minor amounts of lipids, minerals, sugars and free amino acids (FAAs) [4]. Scientific findings reveal that, eventhough found as minor constituents, FAAs together with soluble sugars play a significant role in deciding the organoleptic properties of food [3,5]. Moreover, the presence of the FAA; asparagine in relation to acrylamide, which is a carcenogenic compound formed during heating has been discussed in literature [6,7]. In addition, FAA profile has been successfully used for discrimination of variety and origin of natural foods in food authentication [3,8,9]. Having inspired by these facts, there is a growing concern among the scientific community in researching on the FAA levels present in foods. Apparently, there is an increased demand for analytical methods sensitive in trace level detection.

There are several studies that describe FAA analysis in food [6–12], including rice [3,13–15]. In the methods described in literature, the FAAs are quantified using either high performance liquid chromatography (HPLC) [15–17], gas chromatography (GC) [11,18], capillary electrophoresis [19,20], ion exchange chromatography [21,22], liquid chromatography coupled to mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS/MS) [23,24].

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Owing to the high polarity, low volatility and the absence of specific chromophores for ultraviolet (UV) or fluorescence detection, amino acid analysis generally requires a derivatization step which improves the separation and the sensitivity of detection. Pre-column derivatization using either orthophthaldialdehyde (OPA) [15,16,25,26] or 9-fluorenylmethyl chloroformate (FMOC) [16,17,25,26] reagents are the most common derivatizations found in literature associated with amino acid analysis. However, these techniques generally encounter complexities related to derivatization such as incomplete derivatization, derivative instability, reagent interference, long preparation times, lack of analyte specificity and the hazard associated with the use of potentially toxic derivatizing reagents [27,28]. In addition, the use of costly reagents and buffers, lenghty run times and decreased reproducibility are among the other drawbacks associated with derivatization. In contrary, LC–MS/MS technique enables, analysis of amino acids without derivatization [27–34]. Therefore, the analytical limitations inherent with derivatization are eliminated in the underivatized LC–MS/MS detection with improved selectivity.

Several approaches have been made for underivatized FAA analysis using MS/MS detection combining either hydrophilic interaction liquid chromatography (HILIC) [29–31] or reversed phase liquid chromatography (RPLC) [10,32–34]. Number of studies report the use of HILIC for successful separation of the whole spectrum of underivatized FAAs [29–31]. However, in comparison to HILIC, relatively a lesser number of underivatized FAAs have been reported in relation to RPLC, as the chromatographic methods described have failed to separate the complete profile of amino acids on RPLC [10,32–34]. In addition, less focus has been made on food matrices as only few studies are found that describe underivatized FAA analysis in food using RPLC [10,32]. In the studies described, elution of majority of the analyzed amino acids have occured in the void volume with absolutely no chromatographic resolution, specifically hindering the selective identification of the structural isomers. Therefore, these methods demonstrate less specificity, which is paramount for the accuracy and the reliability of the analytical method towards the amino acids which are structural isomers. On the otherhand, very limited studies have outlined the method performance characteristics performed on cereal matrix [12,15,20], which is vital for any analytical technique to be deemed acceptable. Moreover, all these analytical methods described have several drawbacks including lengthy sample preparation and the downsides associated with the derivatization. Further, none of these analytical methods have demonstrated acceptable method performance characteristics for the full spectrum of amino acids. In this context, the aim of this study was to develop a simple and rapid, RPLC-MS/MS method with acceptable method perfomance characteristics for direct, underivatized analysis of FAAs in rice including amino acids which are structural isomers. The applicability of the method was tested on seven traditional and improved local varieties and two other imported varieties of rice consumed in Sri Lanka.

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), L-hydroxyproline (Hyp), L-lysine (Lys), L-proline (Pro) and L-Norleucine (Nor), each of purity >98% were obtained from Sigma Aldrich Chemicals, Germany and the internal standard; L-Theanine with purity >98% was purchased from Baxter Smith Labs, USA. All the other chemicals used and the solvents were of either LCMS grade or HPLC grade purchased from Sigma Aldrich.

The stock solutions of amino acids; L-Asn, L-Gln, L-Trp, L-Hyp, L-theanine, and L-Nor were prepared in ultra pure water while the rest of the amino acids were prepared in 0.1 M HCl solution. The calibration standard solutions were prepared in a solution which comprised of water/methanol (3:2).

The mobile phases; (A) composed of water/methanol (90:10) with 0.1% (v/v) formic acid while (B) composed of water/ methanol (50:50) with 0.1% (v/v) formic acid.

In order to study the applicability of the method, four Sri Lankan traditional and improved rice varieties: Sooduru samba, Mawee, Bg 406, Bg 38, cultivated under experimental field conditions at Rice research development centers (RRDCs) at Bathalagoda and Bombuwala, three commercially available rice varieties; Keeri samba, Kekulu samba, Suwandel and two imported rice varieties which are commercially available in the country: Basmathi, Ponni were selected for the analysis.

2.2. Sample preparation

The finely ground rice samples were sieved through 0.5 mm sieve. To 0.2 g of the seived rice samples, 100 μL each of the IS's; L-theanine, and L-Nor which were of 100 mg/L concentration were added. The FAAs were extracted by shaking, 0.2 g of the seived rice sample in 10 mL of methanol/water (40:60, v/v) mixture for 10 min in a mechanical shaker at 125 rpm followed by centrifugation at 15,000 rpm for 10 min. The supernatant solution after filtration through 0.22 μm Nylon syringe filter was injected for tandem mass spectrometric detection.

2.3. Instrumentation and analytical LC–MS/MS method

The LC–MS/MS system consisted of Eksigent Expert Ultra LC 100 (Eksigent, Netherlands) UPLC system with a binary pump, coupled to a ABSciex QTrap 4500 series triple quadrupole linear ion trap mass spectrometer (Sciex, USA) in electron spray ionization (ESI) mode. The chromatographic separation was achieved using gradient elution on an Agilent Zorbax Eclipse C18 (4.6 × 100 mm, 5 μm) column. The gradient elution started with 90% A for 0 min; ramped to 30% of B within next 6.5 min at a flow rate of 0.3 mL/min; ramped to 100% of B in 7 min and was kept at 100% of B till 8 min; ramped to 90% A in 8.5 min and kept at -90% of A till 12.5 min at a flow rate of 0.4 mL/min. The column was operated at 40 °C throughout the total runtime which was 12.5 min. The sample injection volume was 3 μL.

The ion spray voltage was set at 5500 V while the source temperature was set at 500 °C. The nebulizer gas and the heater gas were maintained at 50 kPa.

Data acquisition and processing were performed using Analyst software (version 1.6.2) from Sciex Corporation, USA.

2.4. Optimization of the MS/MS conditions

The LC–MS/MS detection was performed in positive mode with multiple reaction monitoring (MRM). The optimization of MRM method parameters was carried out through the direct infusion of analyte mixtures to the mass spectrometer. The ion spray voltage, source temperature, nebulizer gas and the heater gas were optimized to improve the sensitivity of the analytes. The most abundant mass fragment was selected as the quantifier while the second most abundant mass fragment was selected as the qualifier respectively as given in Table 1. Due to the relatively lower masses, only one product ion of the precursor ion was possible with Gly, Ala and Pro, while for amino acids with relatively higher molecular masses such

Table 1
Method conditions for LC–MS/MS analysis.

Amino Acid	RT (min)	ESI Mode	Q* (Da)	Q^1 (Da)	Q^2 (Da)	DP (V)	EP (V)	CE (V)	CXP (V)
Asn	3.00	Pos	133	116	74	31,41	10	13,25	8,22
Hyp	3.07	Pos	132	86	68	36,31	10	17,25	8
Phe	8.33	Pos	166	120	103	31	10	21,35	12,28
Val	3.73	Pos	118	72	55	36	10	13,29	10,6
Lys	3.67	Pos	147	130	84	16,21	10	13,23	6,8
Ala	3.04	Pos	90	44	–	20	10	19	6
Tyr	5.12	Pos	182	165	136,123	46,51	10	19,13,25	6,16
His	2.77	Pos	156	110	93	41	10	17,31	14,8
Trp	9.70	Pos	205	188	146	41	7	15,24	9,39
Ser	2.98	Pos	106	60	42	31	10	15,31	6,4
Asp	3.03	Pos	134	88	70	26,36	10	13,25	14,6
Met	4.20	Pos	150	133	104	21	10	13,15	8,12
Glu	3.10	Pos	148	102	84	21	10	15,23	4,12
Pro	3.33	Pos	116	70	–	8	7	23	9
Gly	2.95	Pos	76	30	–	46	10	17	8
Ile	5.50	Pos	132	86	69	40	7	15,24	14,9
Leu	5.85	Pos	132	86	69	36	10	23	8,20
Arg	2.79	Pos	175	116	70,60	46,56,41	10	19,35,19	6,8,6
Thr	3.06	Pos	120	74	56	46,86	10	15,23	8,6
Gln	4.00	Pos	147	84	102	21,31	10	23,19	6,12
Nor (IS)	6.14	Pos	132	86	68	31	10	25	8
Theanine (IS)	4.10	Pos	175	84	158	35	10	27,16	24,39

RT: Retention time, Q*: Parent mass, Q^1: Quantifier mass, Q^2: Qualifier mass, ESI: Electron Spray Ionization, DP: Declustering potential, CE: Collision energy, EP: Exit potential, CXP: Collision cell exit potential, 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, Lys: Lysine, Trp: Tryptophan, Phe: Phenylalanine, Ile: Isoleucine, Leu: Leucine, Pro: Proline Nor: Norleucine, Hyp: Hydroxyproline, IS: Internal standard.

as Tyr and Arg, three dominant product ions were observed. For the rest of the amino acids, the MRM ratio was assessed using the areas obtained for the two most abundant product fragments.

2.5. Optimization of the liquid chromatographic method

The chromatographic method was optimized using methanol as the organic solvent in the mobile phases. The pH of the mobile phases was varied from neutral to acidic upto methanol/water combinations containing 1% formic acid and it was found that the optimum chromatographic peak shapes and resolution were obtained with mobile phases containing 0.1% formic acid. The steepness of the gradient in introducing of the organic phase, had a significant impact on the separation of the amino acids in the RP phase. It was observed that with less steep gradients, separation was comparatively improved at the cost of broader peak shapes. Increased flow rates aided in obtaining narrower chromatographic peak shapes, but in contrary affected the resolution of isomers. Therefore, the flow rate of the mobile phase and the gradient conditions of the chromatographic method were optimized such that the baseline resolution was achieved for the critical pairs of transitions with the same parent and the product masses such as Hyp/Ile/Leu/Nor isomers (*m/z* 132/86), and Lys/Gln (*m/z* 147/84) as given in Fig. 1, and hence it was assured that the selectivities of the analytes were not impaired.

2.6. Optimization of the FAA extraction

The pH of the extraction solvent and the proportion of methanol in water, were varied to optimize the extraction of FAAs based on recoveries. Both parameters significantly affected the extraction of FAAs which have either acidic moieties in the alkyl group of the amino acid such as Glu and Asp and basic moieties such as Arg, Lys and His while the FAAs with neutral alkyl groups were not significantly affected. The extraction using methanol and water in 2:3 volumetric ratio under neutral conditions exhibited the optimum recoveries for the majority of the analyzed FAAs and hence was used in the extraction step.

2.7. Validation of the method

The method validation protocol was based as per the guidelines described in FDA, AOAC International and Eurachem Method validation guidelines [35–38]. For the validation procedure, the samples fortified with the analytes at three concentration levels (low, mid and high) covering the working range, were analyzed to evaluate the method performance characteristics. Low, mid and high level represent, 20%, 50% and 80% of the working range respectively. Six replicates at each concentration level were separately analyzed for the evaluation. The applicability of the method was tested on nine rice varieties and different cereal matrices.

2.8. Statistical Analysis

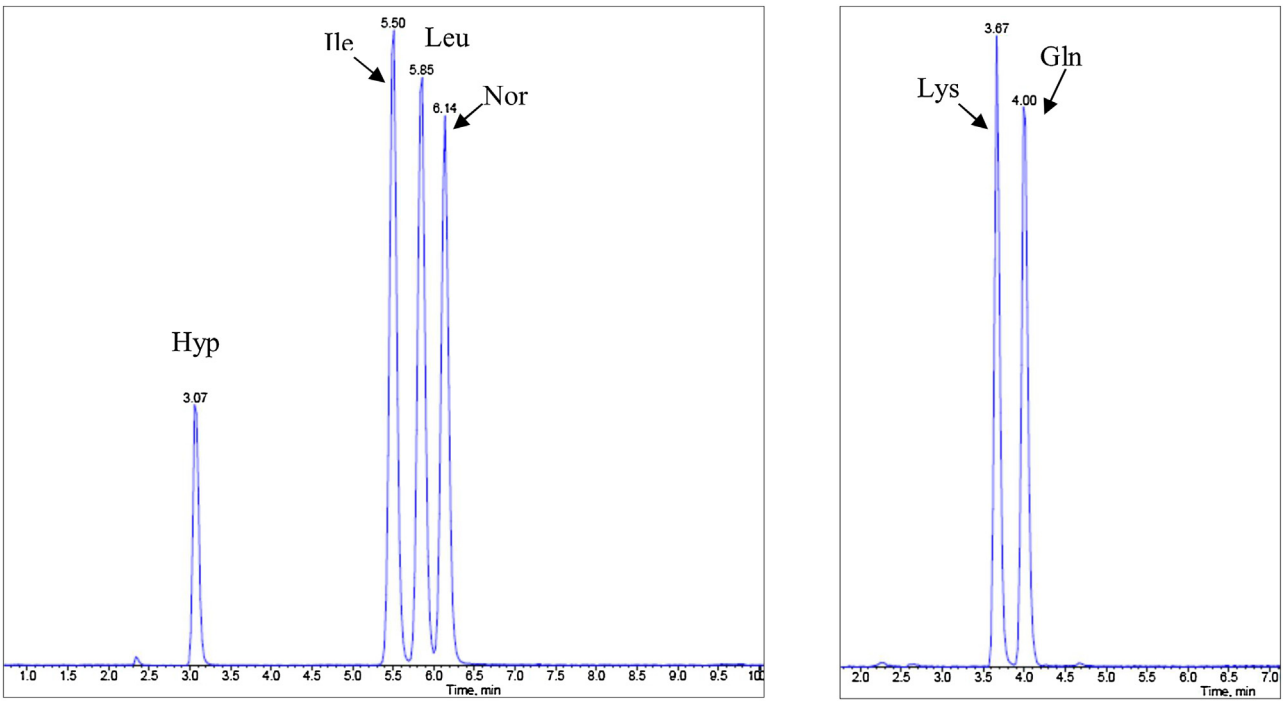
The statistical analysis was performed in Microsoft Excel 2007 at 5% significance level. In evaluation of the method performance characteristics, the percentage relative standard deviations (% RSD) were considered to assess the intra and inter day precision and recovery values. The robustness of the method was evaluated by applying *t*-test to the results obtained after changing of the parameters as per the Plackett Burmann design.

For the expanded uncertainty for each amino acid, the estimation was performed at 95% confidence level, considering the pooled standard deviations arising from the repeatability and reproducibility studies carried out at three concentration levels and the linear regression of the calibration curve. Further, the results obtained for the rice cultivars were statistically analyzed using ANOVA.

3. Results and discussion

3.1. Selectivity

Satisfactory chromatographic resolution of 20 amino acids was achieved using gradient elution. The selectivity of the method was assured considering the qualifier to quantifier area ratio calculated using the mass fragments mentioned in Table 1. A tolerance level of



(a) m/z 132/86) **(b) m/z 147/84**

Fig. 1. Chromatographic resolution of amino acids with identical mass transitions.

±20% was considered acceptable for ion ratio precision, when compared against the ratios observed for the reference standards. For the three amino acids; Gly, Ala and Pro where two transitions were not available, the selectivity was assured by the comparison of the relative retention times calculated with respect to the IS's in the samples to those of reference standards and through fortification studies. For the Val, Ile and Leu, Nor was considered as the IS, and for the rest of the amino acids, L-theanine was considered as the IS [15]. For those amino acids which have identical transitions, such as Hyp, Ile/Leu, the selectivity was assured by the relative retention times calculated relative to their respective IS's. The calculated resolution factors (Rs) for the two isomers; Ile/Leu as well as for the Gln/Lys pair, were greater than 1 demonstrating baseline resolution, indicating better selectivity for all the amino acids analyzed as given in Fig. 1. Therefore, in comparison to the previous work reported on methods describing underivatized FAA detection in food matrices using RPLC-tandem mass spectrometry [10,32], the present study demonstrates better resolution for the Ile/Leu pair as well as for Lys/Gln amino acid pair enabling the analysis of full spectrum of FAAs encountered in a typical chromatographic run with increased selectivity and improved chromatography as given in Fig. 2. In addition, the current chromatographic method enables satisfactory resolution for selective identification of 20 amino acids on RPLC in comparison to the 18 amino acids resolved on RPLC in previous studies in different matrices [33,34].

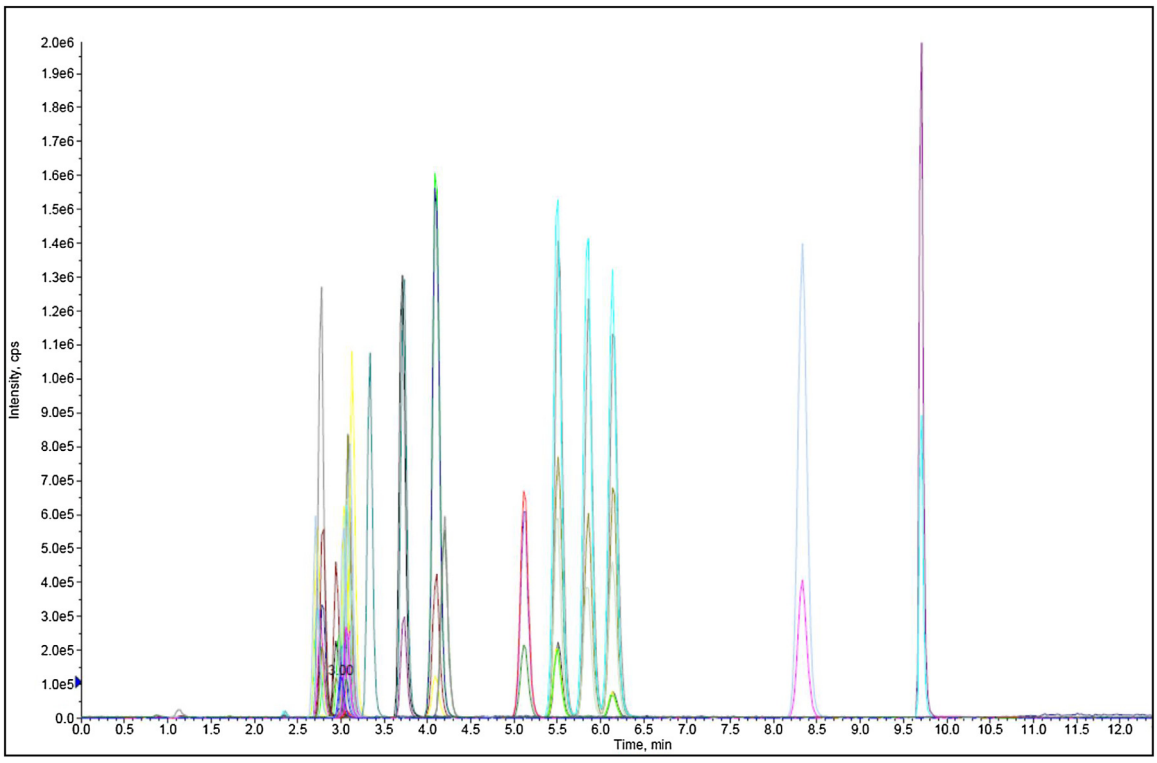
3.2. Matrix Effect

The effect of matrix that results in either ion suppression or ion enhancement is a usual phenomenon experienced in quantitative analysis using LC–MS/MS analysis. Therefore, evaluation of the significance of matrix effect is paramount in accurate quantification using tandem mass spectrometry. The estimation of the matrix effect was performed by considering the ratio between the slopes obtained for the external standard calibration curve to that of the matrix-matched calibration curve. For those amino acids, where the ratios were below 0.90 or above 1.10, it was considered that the matrix effect was associated as either signal suppression or enhancement, respectively. If the values were within that range, allowing a tolerance of 10%, the matrix effect was considered insignificant These ratios obtained for the amino acids are given in Table 2. Out of the studied amino acids, for Lys, Tyr and Arg, the matrix effect was shown significant. Hence for the three amino acids, the method performance characteristics were evaluated based on the values calculated using the matrix-matched calibration curves.

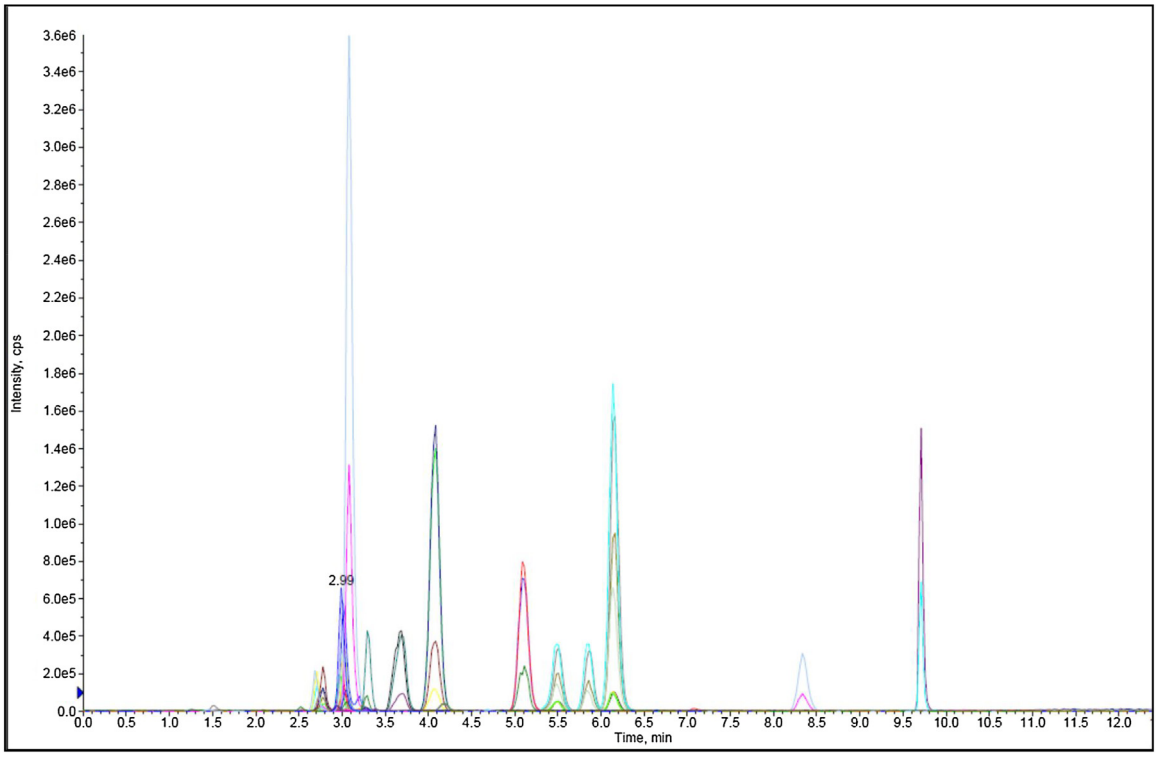
Further, the matrix robustness of the method was tested against several cereal grains; wheat, corn and finger millet by evaluation of the recoveries obtained for the said matrices at fortification in the mid level of the working range. The recovery values of FAAs for the three matrices as mentioned in Table 2, were within 80%–110% for the majority of the amino acids, demonstrating excellent matrix robustness and applicablity of the method over other cereal grain matrices.

3.3. Precision

The precision of the method was measured by analysing six replicates of the sample that contain targeted analytes spiked at three concentration levels (low, mid and high) covering the working range. Fortification of the targeted analytes was ensured to represent 20%, 50% and 80% of the entire working range of the method. The intermediate precision of the method was evaluated under repeatable conditions on the same day by the same analyst (intra-day precision) and under reproducible conditions; by different analysts on different days (inter-day precision) in a random order over a period of four months. The intermediate precision of the method expressed as percentage relative standard deviation (% RSD) of concentrations, were below 10% for all the amino acids analyzed as given in Table 3. These values are in well agreement



(a)



(b)

Fig. 2. LC–MS/MS Profile of a) Amino acid standards; b) Rice sample.

with the predicted RSD values for the specific analyte concentration range calculated from the Horwitz formula which is 11% and 16% for the repeatability and reproducibility respectively [36].

3.4. Accuracy and recovery

The accuracy of the method was evaluated based on recovery studies due to the absence of a certified reference material (CRM)

Table 2
Matrix Effect and matrix robustness.

Amino acid	Calibration equation for normal external standards	Calibration equation for matrix matched standards	Ratio between slopes[a]	% Recovery (n = 3)		
				Wheat	Corn	Finger Millet
Asn	y = 2.93E+03x + 3.08E+04	y = 3.17E+03x + 2.41E+06	0.92	82 ± 6	91 ± 6	98 ± 5
Ala	y = 3.78E+03x − 8.74E+03	y = 4.17E+03x + 3.23E+06	0.91	82 ± 1	81 ± 7	105 ± 1
Lys	y = 1.24E+04x − 1.55E+05	y = 1.99E+04x + 4.38E+05	0.62	94 ± 4	95 ± 2	104 ± 4
Val	y = 2.50E+04x + 2.28E+05	y = 2.58E+04x + 2.12E+06	0.97	108 ± 3	90 ± 7	108 ± 8
Phe	y = 6.62E+04x − 7.06E+05	y = 6.61E+04x + 2.23E+06	1.00	106 ± 4	106 ± 3	110 ± 1
Tyr	y = 1.90E+04x − 1.50E+05	y = 2.18E+04x + 1.19E+06	0.87	97 ± 1	106 ± 7	97 ± 3
Leu	y = 5.37E+04x − 7.44E+05	y = 5.05E+04x + 1.20E+06	1.06	102 ± 1	100 ± 3	99 ± 2
Gly	y = 2.92E+02x + 1.77E+03	y = 3.09E+02x + 7.14E+04	0.94	73 ± 3	89 ± 8	81 ± 3
Pro	y = 1.42E+04x − 1.30E+05	y = 1.58E+04x + 5.88E+05	0.90	85 ± 4	95 ± 6	118 ± 4
Ile	y = 5.37E+04x − 7.44E+05	y = 5.05E+04x + 1.20E+06	1.06	112 ± 7	116 ± 8	118 ± 4
His	y = 3.08E+04x − 2.06E+05	y = 3.35E+04x + 4.16E+05	0.92	75 ± 8	95 ± 3	60 ± 4
Hyp	y = 5.37E+03x + 4.89E+04	y = 5.74E+03x + 5.80E+04	0.94	80 ± 5	93 ± 2	101 ± 4
Glu	y = 1.29E+04x + 1.07E+05	y = 1.30E+04x + 1.34E+07	0.99	60 ± 7	82 ± 6	82 ± 4
Asp	y = 5.75E+03x + 2.01E+04	y = 6.27E+03x + 3.26E+06	0.92	65 ± 8	80 ± 2	79 ± 7
Met	y = 1.19E+04x + 1.78E+04	y = 1.25E+04x + 1.63E+05	0.95	118 ± 3	120 ± 2	120 ± 4
Gln	y = 1.42E+04x + 1.02E+05	y = 1.49E+04x + 1.98E+05	0.96	90 ± 4	82 ± 2	67 ± 4
Ser	y = 6.10E+03x + 6.00E+04	y = 6.74E+03x + 6.08E+05	0.91	83 ± 1	83 ± 7	97 ± 6
Thr	y = 4.47E+03x + 1.08E+04	y = 4.77E+03x + 2.59E+05	0.94	70 ± 3	80 ± 2	86 ± 4
Arg	y = 8.19E+03x − 2.53E+05	y = 1.24E+04x + 1.01E+06	0.66	72 ± 8	101 ± 3	65 ± 6
Trp	y = 1.25E+04x + 1.05E+05	y = 1.38E+04x + 1.42E+07	0.90	76 ± 7	97 ± 8	102 ± 2

Standard deviation of recoveries are given followed by the ± symbol in the respective cells.
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, Pro: Proline, Lys: Lysine, Hyp: Hydroxyproline.
[a] Ratio between the slopes of calibration curve obtained under normal external standard calibration to the slope of calibration curve obtained under matrix matched conditions.

Table 3
Precision and Recovery.

Amino Acid	Precision (% RSD) (n = 6)						% Recovery (n = 6)		
	Intra-batch			Inter-batch					
	Low	Mid	High	Low	Mid	High	Low	Mid	High
Glu	3.1	0.3	0.3	5.2	0.4	0.3	86 ± 9	100 ± 3	103 ± 4
Asp	7.0	1.3	2.0	9.0	1.7	2.2	99 ± 8	98 ± 2	103 ± 6
Asn	2.3	0.1	0.2	4.5	0.2	0.8	92 ± 8	93 ± 2	104 ± 5
Ala	3.0	0.2	0.2	4.4	0.5	0.7	105 ± 8	105 ± 1	107 ± 6
Lys[a]	2.1	2.3	1.3	2.3	2.5	1.5	80 ± 3	85 ± 1	90 ± 2
Val	1.3	1.2	1.4	2.2	2.4	2.4	96 ± 2	93 ± 2	93 ± 1
Phe	2.4	1.0	1.2	2.8	1.8	2.4	96 ± 2	93 ± 1	95 ± 1
Tyr[a]	0.3	0.2	0.1	2.3	1.2	1.5	99 ± 2	99 ± 2	98 ± 2
His	2.6	2.0	1.4	2.9	2.4	1.8	95 ± 1	95 ± 1	101 ± 2
Hyp	1.3	2.4	2.4	1.8	3.0	3.2	93 ± 2	91 ± 1	97 ± 2
Ser	2.2	2.9	2.2	3.2	3.6	2.8	98 ± 1	94 ± 1	100 ± 3
Thr	2.1	2.3	1.8	2.6	4.3	2.8	94 ± 1	91 ± 2	98 ± 1
Pro	1.3	2.1	2.1	1.6	2.0	2.8	96 ± 2	98 ± 1	103 ± 4
Ile	0.8	0.3	1.2	2.8	2.3	2.2	92 ± 1	89 ± 1	92 ± 4
Leu	2.5	2.5	1.5	2.0	2.9	3.5	80 ± 2	80 ± 1	82 ± 5
Gly	1.6	1.2	2.9	1.3	2.2	3.9	104 ± 3	98 ± 2	101 ± 3
Met	1.2	2.8	1.9	3.2	3.2	2.4	99 ± 1	99 ± 1	101 ± 1
Gln	1.7	1.7	2.9	3.7	2.7	4.5	92 ± 2	89 ± 1	97 ± 1
Arg[a]	2.6	1.5	1.3	3.6	2.5	3.3	85 ± 6	87 ± 2	92 ± 2
Trp	2.5	3.6	0.2	3.9	5.6	1.2	106 ± 3	110 ± 5	110 ± 4

RSD: Relative Standard Deviation.
n = Number of replicates.
Standard deviation of recoveries are given followed by the ± symbol in the respective cells.
Low, Mid and High represent, 20%, 50% and 80% levels of the working range of the method respectively.
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, Pro: Proline, Lys: Lysine, Hyp: Hydroxyproline.
[a] Method performance characteristics were evaluated under matrix matched conditions.

on FAAs. Recovery or the fraction of the analyte determined after addition of a known amount of the analyte to a sample was evaluated after spiking at three concentration levels representing 20%, 50% and 80% levels of the entire working range respectively. The overall recoveries observed for the studied amino acids were within 80–110% as given in the Table 3.

There is very limited data available on recovery studies of FAAs performed on cereal matrices [12,15,20]. Omar et al. in his study reports, acceptable recoveries for Pro, Val, Gln, Asn, Ala and Ser in wheat matrix [20]. Unfortunately, the study has not taken into account the critical amino acids like Glu, Asp and Gly which are found as major FAAs in cereal matrices. In a similar study, Mustafa et al. presents the recovery data for Asn, Gly, Ala, Asp, Val, Ser,

Table 4
Working range, Expanded uncertainty, Linearity, LODs and LOQs.

Amino Acid	Working Range (mg/kg)	% Expanded Uncertainty (k = 2)	R^2	Calibration Equation	LOD (mg/kg)	LOQ (mg/kg)
Glu	1.25–100	13	0.999	y = 1.29E+04x + 1.07E+05	0.5	0.7
Asp	1.25–100	13	0.999	y = 5.75E+03x + 2.01E+04	0.6	0.8
Asn	1.25–100	9	0.999	y = 2.93E+03x + 3.08E+04	0.7	0.9
Ala	1.25–100	13	0.999	y = 3.78E+03x − 8.74E+03	0.7	0.9
Lys[a]	1.25–100	4	0.999	y = 1.99E+04x + 4.38E+05	0.9	1.1
Val	1.25–100	4	0.999	y = 2.50E+04x + 2.28E+05	0.7	0.8
Phe	1.25–100	5	0.999	y = 6.62E+04x − 7.06E+05	0.6	0.8
Tyr[a]	1.25–100	4	0.999	y = 2.18E+04x + 1.19E+06	0.7	0.9
His	1.25–100	4	0.999	y = 3.08E+04x − 2.06E+05	0.6	0.8
Hyp	1.25–100	4	0.999	y = 5.37E+03x + 4.89E+04	0.4	0.6
Ser	1.25–100	5	0.999	y = 6.10E+03x + 6.00E+04	0.7	1.0
Thr	1.25–100	6	0.999	y = 4.47E+03x + 1.08E+04	0.6	0.8
Pro	1.25–100	5	0.999	y = 1.42E+04x − 1.30E+05	0.6	0.7
Ile	1.25–100	4	0.999	y = 5.37E+04x − 7.44E+05	0.5	0.7
Leu	1.25–100	5	0.999	y = 5.37E+04x − 7.44E+05	1.0	1.2
Gly	1.25–100	5	0.999	y = 2.92E+02x + 1.77E+03	1.0	1.2
Met	1.25–100	5	0.999	y = 1.19E+04x + 1.78E+04	0.9	1.1
Gln	1.25–100	5	0.999	y = 1.42E+04x + 1.02E+05	0.4	0.6
Arg[a]	1.25–100	6	0.999	y = 1.24E+04x + 1.01E+06	0.9	1.1
Trp	1.25–100	8	0.999	y = 1.25E+04x + 1.05E+05	0.9	1.1

LOD: Limit of Detection, LOQ: Limit of Quantification.
R^2: Regression Coefficient, Number of Replicates (n) = 6.
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, Pro: Proline, Lys: Lysine, Hyp: Hydroxyproline.
[a] Method performance characteristics are evaluated under matrix matched conditions.

Trp and Glu [12]. However their method also fails to cover the whole spectrum of amino acids available in the FAA pool of cereals. Further, the recovery obtained for Glu, which is generally present as the predominant FAA in rice, exceeds the acceptable recovery range specified for the analyte concentration which is 80–110% as recommended by the FDA Guidelines for the validations of Chemical Methods for the Foods Program and the AOAC Guideline for Standard Method Performance Requirements [35–38], while the recovery reported for Trp in the same study is below the acceptable level.

3.5. Limit of detection and limit of quantitation

The minimum amount that can be detected or the LOD and the minimum detectable values that can be quantified with confidence or the LOQ, were established based on the mean and the standard deviation values obtained for six independent replicates of blank samples spiked at lowest detectable concentrations. To the mean value obtained for the blank response fortified at lowest detectable concentrations, approximately 3 times and 5 times of the standard deviation of the response detected for the fortified blanks were added to give the LOD and LOQ values respectively. The LODs and the LOQs for the amino acids calculated using the aforementioned method were in the range 0.4–1.0 mg/kg and 0.6–1.2 mg/kg respectively allowing high sensitivity in detection of FAAs which are present in trace levels. The lowest LOD and LOQ values observed were for Hyp and Gln, whereas the highest LOQ values were observed was for Gly and Leu respectively as given in Table 4. In comparison to previous method validation studies perfomed on cereal matrices [15], except for Gly and Val, the developed analytical method has comparatively lower LOD and LOQ values than the LOD and LOQ values previously reported, allowing higher sensitivity in detection.

3.6. Linearity

The calibration range consisted of seven calibration levels at 25, 50, 100, 500, 1000, 1500 and 2000 μg/L. The calibration curves were constructed by fitting the analyte concentrations of the calibrators versus the peak area ratios of the analyte to IS with line regression. The regression coefficient, R^2 determined from the calibration curves were greater than 0.999 as given in Table 4 for all the amino acids analyzed indicating higher linearity for the method over a wide working range spanning from 1.25 mg/kg–100 mg/kg

3.7. Measurement uncertainty

The percentage expanded uncertainties were calculated for all the studied amino acids considering the uncertainties arising from standard preparation, sample preparation, precision and regression associated with calibration. The percentage expanded uncertainties evaluated based on low, mid and high analytical levels representing the entire working range were considered and the highest uncertianties observed for each amino acid were reported. The uncertainties for each amino acid calculated as per the guide [39], considering the factors contributing to the final result with a coverage factor of 2 (k = 2) were below 14% for all the amino acids analyzed as given in Table 4. Customarily, the uncertainties arising from the precision of the method and the regression analysis involved in the calibration step, constituted as the major contributory factors to the final percentage uncertainty.

3.8. Robustness of the method

Robustness of the method was assessed considering deliberate changes made to seven variables; extraction speed, extraction time, composition of extraction solution, composition of the mobile phases, pH of the mobile phase, gradient conditions employed and the flow rate of the gradient run, as per the Placket Burmann design. When student's *t*-test was performed on the results, it was observed that the composition of the extraction solution, pH of the extraction solution, extraction speed and the duration of the extraction significantly influenced the recoveries of FAAs. In addition, changes in the pH of the mobile phase, gradient conditions, flow rates employed, casued significant changes to chromatographic separation hindering resolution of isomers of Gln/Lys and Ile/Leu/Nor. Therefore in

FAA analysis, factors affecting the extraction and resolution need to be precisely controlled for the generation of accurate and reproducible results.

The extraction procedure described in the study uses no clean up step. However, during the extraction step, removal of the co-extracted compounds such as proteins were effectively carried out through the deproteinization involved with the use of organic solvent; methanol followed by centrifugation performed at higher rpm values. These steps are vital to remove the matrix interference in detection and to prevent the progressive clogging of the column by co-extracted macro molecules to assure reproducible results.

In comparison to lengthy and laborious sample preparations involved in previous studies [12,15], the developed extraction procedure outlined in this method involves least number of steps incorporating less reagents allowing maximum recovery of analytes and hence is quick, easy and cost effective. When this extraction procedure is combined with the underivatized MS/MS detection performed on RPLC with short run time, the analytical method enables extremely rapid, high throughput FAA analysis.The method performance characteristics summarized in this study comply with the requirements specified for specific concentration levels as defined by the international guidelines on method validation [35–38] and serve as one of the very limited comprehensive method validation work carried out using tandem mass spectrometric detection on RP phase enabling direct analysis of 20 FAAs in rice and hence is a valuable addition in the field of chemical analysis related to food industry.

3.9. Application of the validated method to rice samples

The Table 5 summarises the FAAs levels determined using the validated method in nine rice varieties grown at the Bathalagoda and Bombuwala RRDCs of Sri Lanka and rice varieties which are widely consumed in the country collected from the open economic centers. As observed in simillar studies carried out in other parts of the world including Sri Lanka [3,13–15], Glu, Asp, Asn and Ala continue to be the predominant FAAs reported in the experimented rice cultivars which are known to be significantly contributing to the taste of rice, while His and Hyp being the least present. Statistical analysis done using ANOVA on the FAA profiles of each rice cultivar, provides evidence that the FAA composition to be significantly different with respect to the cultivar and indicates the possiblity of use of FAA profile for screening of variety. However, further studies which accounts the effect on the FAA composition from agricultural practices, climatic conditions and development controls are required to establish the specific correlations and validate such findings.

4. Conclusion

The study presents a simple, rapid and a sensitive method developed and validated for underivatized analysis of FAAs in rice using RPLC-MS/MS. The recoveries suggest the applicability of the method for other cereal matrices as well. The validated method is accurate, reproducible, and complies with the acceptance criteria required in the method validation guidelines allowing determination of FAA in rice present in trace levels. Hence, the method will serve as a reliable tool to evaluate the FAA composition in rice and other cereal matrices. Further, the advent of simple, rapid and reliable analytical techniques for analysis of FAA profiles as described in the present study, will create new scientific perspectives in future investigations related to FAA profiling of food with respect to authentication and organoleptic characterization.

Table 5
Free amino acid levels of selected rice cultivars consumed in Sri Lanka.

Cultivar Type		Free Amino acid content (mg/100 g), dry basis (n = 3)																			
		Asn	Hyp	Phe	Val	Lys	Ala	Ile	Tyr	His	Ser	Asp	Met	Glu	Pro	Gly	Thr	Gln	Leu	Trp	Arg	Total
Mawee	Mean	2.02	0.20	0.28	1.86	0.20	1.45	0.68	0.41	0.47	0.52	3.80	0.26	3.22	0.47	0.61	0.61	0.69	ND[c]	8.15	0.43	26.33
	SD	0.08	0.01	0.01	0.20	0.02	0.18	0.05	0.01	0.01	0.05	0.30	0.01	0.13	0.04	0.09	0.04	0.01	–	0.72	0.01	1.97
Suwandal	Mean	2.70	0.16	0.27	2.79	0.42	5.69	0.88	0.16	0.30	0.68	2.16	ND[b]	2.99	0.42	0.99	0.56	0.47	ND[c]	0.56	0.43	22.63
	SD	0.27	0.01	0.01	0.14	0.04	0.32	0.08	0.04	0.01	0.22	0.21	–	0.34	0.04	0.19	0.05	0.02	–	0.02	0.02	2.03
Sooduru samba	Mean	6.20	0.20	0.40	1.00	1.20	7.10	0.50	1.00	0.60	2.30	5.00	0.30	5.90	1.20	4.40	1.90	0.10	0.60	0.69	1.50	42.09
	SD	0.32	0.03	0.03	0.08	0.07	0.45	0.03	0.11	0.05	0.18	0.43	0.02	0.39	0.07	0.30	0.14	0.03	0.06	0.02	0.11	2.92
Bg 38	Mean	3.56	0.19	0.28	2.59	0.23	2.49	0.90	0.32	0.35	0.87	3.70	0.62	3.52	0.71	1.38	0.76	0.73	ND[c]	0.53	0.67	24.40
	SD	0.11	0.01	0.03	0.29	0.02	0.24	0.14	0.07	0.02	0.06	0.09	0.09	0.23	0.07	0.26	0.04	0.04	–	0.05	0.09	1.95
Bg 406	Mean	3.66	0.26	0.28	2.81	0.16	6.53	0.88	0.31	0.32	0.80	3.50	5.05	2.02	0.57	1.83	0.72	0.58	ND[c]	0.46	0.35	31.09
	SD	0.29	0.01	0.03	0.27	0.00	0.49	0.12	0.05	0.01	0.05	0.17	0.03	0.36	0.06	0.17	0.07	0.05	–	0.06	0.01	2.3
Keeri samba	Mean	3.85	0.22	1.46	9.71	2.94	9.20	3.45	2.15	0.82	1.25	1.57	1.69	4.30	1.78	2.03	1.65	1.75	2.09	1.39	2.10	55.40
	SD	0.20	0.01	0.14	0.40	0.11	0.26	0.29	0.35	0.05	0.14	0.11	0.09	0.25	0.10	0.03	0.07	0.11	0.03	0.08	0.11	2.93
Kekulu samba	Mean	1.87	0.16	0.25	1.53	0.19	1.86	0.44	ND[a]	0.24	0.04	0.75	0.15	2.20	0.50	0.22	0.36	0.47	ND[c]	0.32	0.26	11.81
	SD	0.17	0.01	0.01	0.20	0.00	0.12	0.05	–	0.00	0.04	0.08	0.01	0.21	0.04	0.09	0.04	0.02	–	0.01	0.03	1.13
Basmathi	Mean	4.06	0.16	0.37	2.10	0.37	1.14	0.61	0.12	0.29	0.56	1.21	0.27	3.24	0.50	0.77	0.45	0.45	ND[c]	0.17	0.21	17.05
	SD	0.25	0.02	0.01	0.06	0.05	0.09	0.01	0.02	0.03	0.04	0.14	0.04	0.37	0.06	0.08	0.01	0.02	–	0.02	0.02	1.34
Ponni	Mean	3.70	0.14	0.38	1.95	0.24	1.54	0.68	0.22	0.35	0.37	1.21	0.37	0.99	0.43	0.47	0.36	0.51	0.21	0.60	0.67	15.39
	SD	0.12	0.01	0.01	0.10	0.08	0.18	0.08	0.04	0.02	0.09	0.19	0.04	0.06	0.07	0.19	0.03	0.03	0.02	0.04	0.04	1.44

SD: Standard Deviation, ND: Not Detected, 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, Leu: Leucine, Trp: Tryptophan, Phe: Phenylalanine, Ile: Isoleucine, Pro: Proline, Lys: Lysine, Hyp: Hydroxyproline.

[a] Limit of Quantification: 0.09 mg/100 g.
[b] Limit of Quantification: 0.11 mg/100 g.
[c] Limit of Quantification: 0.12 mg/100 g.

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