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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